# INTERACTION OF p27(KIP1) WITH FKBP-12

## GRANT SUPPORT

This invention was made with United States Government support under grant number 70NANB5H1066 awarded by the National Institute of Standards and Technology. Accordingly, the United States Government has certain rights in the invention.

## FIELD OF THE INVENTION

The present invention is directed to complexes of p27(Kip1) and FKBP-12 proteins. In addition, the present invention relates to the production of antibodies to the aforementioned protein complex, and its use in, *inter alia*, screening, diagnosis, prognosis and therapy.

## BACKGROUND OF THE INVENTION

Loss of control of cell proliferation may lead to severe diseases and disorders (e.g., neoplasia). Hence, the elucidation of the intricacies of the cell-cycle, and its deregulation during oncogenesis, will provide novel opportunities in the prophylactic, diagnostic and therapeutic management of cancer and other proliferation-related diseases. A better understanding of the cell-cycle could be achieved by the elucidation of the interactions of the various protein complexes whose levels and biological activities are regulated through the cell-cycle. The identification and classification of these protein complexes will be useful in the development of treatment modalities and assays for various pathological processes including, but not limited to, hyperproliferative disorders (e.g., tumorigenesis and tumor progression), as well as other related genetic disorders.

It should be noted that the citation of a reference herein should not be construed as an admission that such is prior art to the present invention.

### p27(KIP1)

#### Role of p27(Kip1) in the eukaryotic cell cycle

Eukaryotic cell cycle progression is controlled by the activation and inactivation of a highly conserved family of protein complexes. The protein complex minimally consists of a catalytic subunit having kinase activity (cyclin-dependent kinase or CDK) and a regulatory subunit (cyclin). Each phase of the cell cycle is characterized by the expression of a unique profile of such cyclin-CDK complexes.

For instance, the commitment of cells to enter the DNA synthesis (S) phase of the cell cycle occurs at a restriction (R) point late in the first gap (G1) phase of the cell cycle. Progression through this first gap phase is regulated by the activity of the D-type cyclins and cyclin E, which associate with CDK4 and CDK6, respectively. Sherr & Roberts, 1995, Genes and Dev. 9: 1149-1163.

CDK inhibitors are negative regulatory proteins that bind to a cyclin-CDK complex and inhibit their catalytic activity. Sherr & Roberts, 1995, Genes and Dev. 9: 1149-1163. Two families of low molecular weight proteins that are CDK inhibitors are the Ink4 family and the Cip1/Kip1 family of proteins. The Cip1/Kip1 family of proteins, which includes p21(Cip1), p27(Kip1), and p57(Kip2), have broad specificity and potently inhibit the activity of most cyclin-CDK complexes. Cip1/Kip1 proteins appear to regulate cell proliferation by stopping cell cycle progression in response to a variety of anti-mitogenic signals. The importance of these cyclin inhibitor proteins is evidenced by the fact that cancer development and/or progression is strictly linked to alterations of molecular mechanisms controlling the cell division cycle.

Human p27(Kip1) (27 kDa kinase inhibitor protein), encodes a 22 kDa protein of 198 amino acids and is broadly expressed in human tissues with similar p27(Kip1) encoding mRNA levels in both proliferating and quiescent cells. Polyak *et al.*, 1994, Cell 78: 59-66; Toyoshima and Hunter, 1994, Cell 78: 67-74; U.S. Patent No. 5,688,665. The nucleotide sequence is available in GenBank under Accession No. U10906. p27(Kip1) appears to be primarily responsible for regulating CDK activity by inhibiting cyclin-CDK complex-associated kinase activity (for example, cyclin D-CDK4, cyclin E-CDK2, and cyclin A-CDK2) in response to extracellular antiproliferative cues, thereby arresting the cell-cycle and preventing proliferation. Nomura *et al.*, 1997 Gene 191: 211-218. The involvement of p27(Kip1) in the negative regulation of cell proliferation suggests that it also functions as a tumor suppressor gene. In addition to its role as an inhibitor, p27(Kip1) may function as an adaptor protein to facilitate assembly of specific CDK/cyclin complexes that will have specific functions. LaBaer *et al.*, 1997, Genes and Dev. 11: 847-862.

**Functional domains of p27(Kip1)**

Several functional domains of p27(Kip1) have been identified. One such domain is an amino-terminal domain of 60 amino acids that is both necessary and sufficient for cyclin/CDK complex binding and inhibition. Within this domain, a minimal inhibitory region responsible for inhibiting cyclin-CDK associated kinase activity comprises amino acid residues 28-79. Polyak *et al.*, 1994, Cell 78: 59-66; Toyoshima and Hunter, 1994, Cell 78: 67-74; Kwon *et al.*, 1996, Biochem. Biophys. Res. Comm. 220: 703-709.

Another domain at the amino-terminal region of p27(Kip1) is a cyclin binding motif. Cyclin binding motifs are also found in other CDK inhibitors and is also found in other proteins, including retinoblastoma gene family members p207 and p130, and transcription factors E2F-1, E2F-2, and E2F-3.

The carboxyl-terminal domain of p27(Kip1) can bind cyclin D1 *in vitro*, suggesting that p27(Kip1) associates with D-type cyclins independently of CDK4. Further, is has been demonstrated that CDC2 kinase activity is down-regulated by the carboxyl-terminal region of p27(Kip1). Also, amino acid residue Thr187 located in the carboxyl-terminal domain of p27(Kip1) is a potential substrate site for cyclin-CDK phosphorylation. Vlach *et al.*, 1997, EMBO J. 16: 5334-5344.

It has been demonstrated that the oncogenic adenovirus protein E1A binds to p27(Kip1), however, the interacting domain in p27(Kip1) has not been identified. Mal 1996, Nature 380: 262-265.

**Regulation of p27(Kip1) expression**

In normal cells, expression of p27(Kip1) increases during entry into a quiescent or nondividing state, and rapidly decreases upon re-entry into the cell cycle after stimulation with specific growth factor(s). The abundance of the p27(Kip1) protein is believed to be regulated mainly by translational and post-translational control mechanisms, although some regulation is seen on the transcriptional level.

**(i) Transcriptional Regulation**

Transcriptional regulation of the p27(Kip1) gene may be involved in cellular differentiation. This is indicated by the fact that p27(Kip1) mRNA is downregulated by 1,25-dihydroxyvitamin D3 (Vitamin D3). Vitamin D3 acts through its cognate nuclear receptor (Vitamin D3 receptor) to induce a myeloid leukemic cell line to terminally differentiate into monocytes/macrophages; overexpression of p27(Kip1) directly leads to a terminal differentiation program in these myeloid cells. Liu, 1996, Genes Dev 10: 142-153; Wang *et al.*, 1997, Cancer Res 57: 2851-2855. Furthermore, the mitogen Interleukin-2 also influences the function of the p27(Kip1) gene promoter, which effectively results in the transcriptional down-regulation of p27(Kip1), which results in a significant reduction of p27(Kip1) protein, and thus, the cells are stimulated to progress through the cell cycle. Kwon *et al.*, 1997, J. Immunol. 158: 5642-5648. Nevertheless, mRNA levels of p27(Kip1) are relatively constant in pituitary adenomas and carcinomas, suggesting that p27(Kip1) protein levels are mainly regulated by translational and post-translational mechanisms.

**(ii) Translational and post-translational**

p27(Kip1) is expressed at high levels in hepatoma cells, macrophages, fibroblasts, T-lymphocytes, astroglial cells, and mesanglial cells, and this high level of expression is decreased in response to insulin (Mann *et al.*, 1997, Oncogene 14: 1759-1766), colony-stimulating factor 1 (Antonov *et al.*, 1997, J. Clin. Invest. 99: 2867-2876), serum (Dietrich *et al.*, 1997 Oncogene 15: 2743-2747), Interleukin-2 (IL-2) (Dumont, 1996, Life Sci. 58: 373-395), Interleukin-4 (IL-4) (Liu *et al.*, 1997, J. Imm. 159: 812-819), and platelet-derived growth factor (PDGF) (Shankland, 1997, Kidney Int. 51: 1088-1099).

Other events that regulate p27(Kip1) expression include mitogen deprivation, cell-cell contact, and addition of transforming growth factor (TGF)-β or p53 to a p27(Kip1) expressing cell. The down-regulation of p27(Kip1) by mitogens occurs mainly through the ubiquitin-dependent post-translational degradation pathway. Pagano *et al.*, 1995, Science 269: 682-685; Esposito *et al.*, 1997 Cancer Res. 57: 3381-3385. Also, the phosphorylation of p27(Kip1) by CDK2 at the carboxyl-terminal CDK target site of amino acid residues 187-190 (TPKK) is essential for this post-translational degradation.

When T-cells are stimulated with IL-2, cyclin E-CDK2 complexes become activated and phosphorylate p27(Kip1). The phosphorylated p27(Kip1) is eliminated via the ubiquitin-dependent pathway and the cell cycle progresses. This activation of the cell cycle by degradation of p27(Kip1) can be prevented by the immunosuppressant drug rapamycin, which inhibits the enzymatic activity of cyclin E-CDK2 and results in the presence of abnormally high levels of p27(Kip1) in rapamycin treated cells. Consistent with this mechanism, fibroblasts and T-lymphocytes with a targeted disruption of the p27(Kip1) gene display impaired growth-inhibitory responses to rapamycin. Luo *et al.*, 1996, Mol. Cell. Biol. 16: 6744-6751. The antiproliferative effect of rapamycin is mediated indirectly and only after binding of rapamycin to its receptor FKBP-12 (see below).

Similarly to regulation of p27(Kip1) by rapamycin, transforming growth factor-β (TGF-β) can control the cell cycle by regulating normal and neoplastic cell function and by regulating the expression of various proteins, including p27(Kip1). Jin, 1997, Am. J. Path. 151: 509-519; Polyak *et al.*, 1994, Genes Dev 8: 9-22. TGF-β disrupts the signaling pathway that coordinates the G1 to S phase transition in the cell cycle through several mechanisms, including the upregulation of p27(Kip1) which results in the inhibition of activation of cyclin D-CDK4 complex activity, and inhibition of cyclin E-CDK2 complex activity resulting in the hypophosphorylation of Rb protein. TGF-β inhibition can be reversed by the oncogenic adenovirus protein E1A, which binds to and thereby inhibits the activity of p27(Kip1), such that cells can proceed though the cell cycle. Mal *et al.*, 1996, Nature 380: 262-265; Nomura *et al.*,

1997, Gene 1991: 211-218; Carneiro *et al.*, 1998, Oncogene 16(11): 1455-1465; Muller *et al.*, 1997, Oncogene 15(21): 2561-2576.

Hengst and Reed, 1996, Science 271: 1861-1864 showed that p27(Kip1) protein levels vary throughout the cell cycle; however, the levels of p27(Kip1)-encoding messenger RNA remained constant throughout the cell cycle, and concluded that translational control of p27(Kip1) is an important mechanism for controlling p27(Kip1) protein levels.

In summary, transcriptional, translational and post-translational regulation of p27(Kip1) provide a critical mechanistic link between mitogenic signals and cell cycle progression.

**Role of p27(Kip1) in tumorigenesis and tumor suppression**

Through their roles in cell cycle control, CDK inhibitors such as p27(Kip1) play significant roles in various biological phenomena such as cancer development and/or progression, neuronal differentiation, and apoptosis. Cancer development and/or progression is strictly linked to alterations of molecular mechanisms controlling the cell division cycle. p27(Kip1) regulates the progression through the G1 phase of the cell cycle and at the G1/S phase transition. Thus, p27(Kip1) is implicated in numerous cancers, including leukemia, lymphoma, breast cancer, pancreatic cancer, colorectal cancer, and lung cancer.

Mice lacking p27(Kip1), referred to as "p27(Kip1)(-/-)" herein, are larger than control animals, with thymus, pituitary and adrenal glands, and gonadal organs exhibiting striking enlargement. This is the result of increased numbers of cells in all tissues and organs and confirms the importance of p27(Kip1) in the control of cell proliferation. Similar to mice with a retinoblastoma (Rb) gene mutation, the p27(Kip1)(-/-) mice often develop pituitary tumors spontaneously. This clearly shows that p27(Kip1) plays an important role in inhibiting tumor formation and that p27(Kip1) may act as a growth regulator of a variety of cells.

Despite tumor development in p27(Kip1)(-/-) mice, the p27(Kip1) gene has never been observed to be inactivated in human tumors, and mutations in p27(Kip1) have been detected only in rare cases of primary adult T cell leukemia, non-Hodgkin lymphoma and human breast carcinoma. Hatta *et al.*, 1997, Leukemia 11: 984-989; Ferrando, 1996, Human Genetics, 97: 91-94. Moreover, reduced levels of p27(Kip1) predict poor survival of patients with breast, colorectal and pancreatic cancer. Fredersdorf *et al.*, 1997, Proc. Nat. Acad. Sci. USA 94: 6380-6385; Groshong *et al.*, 1997, Mol. Endocrinol. 11: 1593-1607; Yasui *et al.*, 1997, Japanese J. Cancer Res. 88: 625-629; Kawa *et al.*, 1997, Int. J. Cancer 72: 906-911. p27(Kip1) expression levels correlate with cancer progression since a decrease in p27(Kip1) expression levels significantly correlates with advanced stage, depth of tumor invasion and lymph node

metastasis. Thus, p27(Kip1) is directly implicated in human cancer and expression levels of p27(Kip1) can serve as a useful prognostic marker in cancer.

**Role of p27(Kip1) in differentiation**

p27(Kip1) has been observed to be involved in the differentiation of a number of cell types. For example, the introduction of p27(Kip1) into neuronal, hematopoietic, and muscle precursor cells accelerates their differentiation. Kranenburg *et al.*, 1995, J. Chem. Biol. 87: 1225-1235; Liu *et al.*, 1996, Genes and Dev. 10: 142-153; Guo *et al.*, 1995, Mol. Cell. Biol. 15: 3823-3829. Further, p27(Kip1) was found to be down-regulated in a subset of developing thymocytes (Hoffman *et al.*, 1996 Genes and Dev. 9: 948-962) and high levels of p27(Kip1) accumulate in cortical post-mitotic neurons during mouse neurogenesis whereas low levels were found in their progenitor neuroblasts. Also, elevated levels of p27(Kip1) in staged embryo brain extracts correlate with binding of p27(Kip1) to CDK2. Lee *et al.*, 1996, Proc. Nat. Acad. Sci. USA 93: 3259-3263. p27(Kip1) mediates the withdrawal of oligodendrocyte progenitor cells (0-2A) from the cell cycle during development of the central nervous system, and accumulation of p27(Kip1) in these progenitor cells correlates with differentiation of oligodendrocytes. Casaccia-Bonnefil *et al.*, 1997, Genes and Dev. 11: 2335-2346.

**Role of p27(Kip1) in apoptosis**

During hormone-induced apoptosis, expression of p27(Kip1) increases, and as a result, the G2/M phase transition of the cell cycle is blocked. Furuya *et al.*, 1997, Anticancer Res. 17: 2089-2093. Similarly, growth arrest in anti-IgM induced B-cell lymphomas is dependent on increased synthesis of p27(Kip1). Generally, increased levels of p27(Kip1) correlate with reduced phosphorylation of the retinoblastoma gene product, which leads to cell cycle arrest and subsequent apoptosis. Scott *et al.*, 1997, Curr. Top. Microbiol. Immunol. 224: 103-112; Eyhevesky *et al.*, 1996, Mol. Biol. Cell. 7: 553-564. This is in contrast to that seen in tumor proliferation where p27(Kip1) levels are significantly decreased.

**Role of p27(Kip1) in atherosclerosis**

During atherosclerosis and re-stenosis, abnormal proliferation of vascular smooth muscle cells (VSMC) contribute to intimal hyperplasia. The downregulation of CDK2 activity in these cells is mediated by CDK2-p27(Kip1) complexes. Chen *et al.*, 1997, J. Clin. Invest. 99: 2334-2341, see also US patent 5,672,508. Further, accumulation of monocyte-derived macrophages, which accumulation also contributes to plaque formation, is driven by macrophage colony stimulating factor (MCSF) present in

atherosclerotic plaques. Interestingly, MCSF is required for successful down-regulation of p27(Kip1) before cell cycling. Antonov *et al.*, 1997, J. Clin. Invest. 99: 2867-2876.

**Role of p27(Kip1) in membranous nephropathy**

In progressive glomerulonephritis, the thickening of glomerular mesanglial cells is associated with a marked up-regulation in expression of cyclin kinase inhibitors p27(Kip1) and p21(Cip1). Shankland *et al.*, 1997, Kidney Int. 52: 404-413. Such up-regulation of expression of these kinase inhibitors leads to cell cycle arrest and results in decreased cell proliferation, reduced glomerular function, and resultant renal insufficiency.

In summary, p27(Kip1) is implicated in the control of cell cycle progression, and thus, has a role in tumorigenesis, tumor progression and spread, neuronal differentiation, apoptosis, atherosclerosis, and nephropathy.

**FKBP-12**

The low molecular weight (11.8 kDa) cytosolic drug-binding protein FKBP-12 catalyzes the slow cis to trans isomerization of an Xaa-proline peptide bond in short synthetic peptides. Siehkierka *et al.*, 1989, Nature 341: 755-757; Galat, 1993, Eur. J. Biochem. 216: 689-707. It has thus been classified as a peptidyl-prolyl-cis-trans isomerase (PPIase). Other proteins in this class are the cyclosporin A–binding proteins or cyclophilins. The similar enzymatic activity of FKBP-12 and cyclophilins, together with their ability to serve as receptors for immunosuppressive agents, has justified the generic denomination of "immunophilins" for these proteins. Marks, 1996, Phys. Reviews 76: 631-649.

FKBP-12 binds to the immunosuppressant FK506, hence the name FK506-binding protein. Also, FKBP-12 selectively binds with equivalent affinity to another potent and clinically useful immunosuppressant, rapamycin, and probably mediates rapamycin-dependent immunosuppression. Both FK506 and rapamycin have realized or potential clinical applications in the prevention of graft rejection after organ transplantation and the treatment of autoimmune disorders.

FKBP-12 has been isolated from calf thymus, human spleen (Harding *et al.*, 1989, Nature 341: 761-763) and T-lymphoma cells (Siekierka *et al.*, 1989, Nature 341: 755-757) and was first cloned by Standaert *et al.*, 1990, Nature 346: 641-674 and Maki *et al.*, 1990, Proc. Nat. Acad. Sci. USA 87: 5440-5443. The nucleotide sequence of FKBP-12 is available in GenBank under Accession No. X55741

X-ray crystallography studies have revealed that FKBP-12 has a compact globular structure, containing five anti-parallel beta-sheets that wrap around a short alpha-helix, amino acid residues 58-64.

The immunosuppressant drugs rapamycin or FK506 bind in an oval-shaped deep hydrophobic pocket (containing Tyr26, Phe46, Phe99, Val55, Ile56, Trp59) between beta sheets 3 and 4 and the helix and make contact with the protein through hydrophobic interactions and intermolecular hydrogen bonds, thereby forming a unique effector molecular complex. Whereas the FKBP-12•FK506 complex interacts with and inhibits a $Ca^{2+}$-dependent serine-threonine phosphatase (calcineurin), the FKBP-12•rapamycin complex affects unique biochemical processes of cytokine-mediated signal transduction and blocks the transition from G1 to S phase in the cell cycle.

FKBP-12 is also critical to intracellular $Ca^{2+}$ regulation through effects on the ryanodine and inositol-triphosphate receptors that control calcium-efflux from the sarcoplasmic and endoplasmic reticulum. FKBP-12 is physically associated with and modulates the function of the major $Ca^{2+}$ release channel/ryanodine receptor of the sarcoplasmic reticulum of skeletal and cardiac muscles. The FKBP-12•FK506 complex specifically binds to and inhibits calcineurin, a $Ca^{2+}$ and calmodulin binding signaling protein required for transcriptional activation of the interleukin-2 gene in response to T-cell antigen receptor engagement. Abraham & Wiederrecht, 1996, Ann. Rev. Imm. 14: 483-510. FKBP-12 was also found to be an integral component of the intracellular calcium-release channel complex and can modulate the function of these channels by effecting the channel gating. Brillantes *et al.*, 1994, Cell 77: 513-323.

FKBP-12 also interacts with the type I receptor for transforming growth factor-β (TGF-β RI) and inhibits its signaling function. Wang *et al.*, 1996, Cell 86: 435-444. FKBP-12 binding to TGF-β receptor involves the rapamycin/Leu-Pro binding pocket of FKBP-12 and a Leu-Pro sequence located next to the activating phosphorylation sites in the TGF-β receptor I. This interaction is competitively inhibited by excess FK506; similarly, rapamycin competes with the binding of FKBP-12 to TGF-β receptor. Chen *et al.*, 1997, EMBO J 16: 3866-3876. It is believed that FKBP-12 binding is inhibitory to the signaling pathways of the TGF-β family ligands.

Finally, a distinct function of rapamycin is the involvement of FKBP-12 in ligand-activated immunosuppression and inhibition of cellular proliferation. During the binding of rapamycin to its cytosolic receptor FKBP-12, several biochemical alterations in the cell are mediated. The potent antiproliferative activity of rapamycin involves binding to FKBP-12, and subsequent interaction with targets of rapamycin, resulting in the inhibition of p70S6 kinase. However, neither p70S6 kinase inhibition, nor p27(Kip1)-induced cyclin E-CDK2 inhibition are directly mediated by the FKBP-rapamycin complex. Instead this complex physically interacts with the mTOR protein that has sequence homology with the catalytic domain of phosphatidylinositol kinases. Dumont and Su, 1996, Life Sci. 58: 373-395. According to the linear pathway hypothesis, mTOR affects p27(Kip1) levels and

G1 phase CDKs by modulating the activity of p70S6 kinase on protein synthesis or certain transcriptional events.

In summary, FKBP-12 is implicated in the control of cell cycle progression in various biological phenomena such as tumorigenesis, and tumor progression and spread. Furthermore, FKBP-12 is involved in immunosuppression and may have significant roles in organ transplantation and autoimmune diseases. Also, FKBP-12 is involved in the regulation of calcium-efflux in cardiac and skeletal muscles. In addition, FKBP-12 plays a role in cytokine-mediated signal transduction.

As outlined above, p27(Kip1) and FKBP-12 have been described to be involved in similar processes. However, no direct association or interaction of p27(Kip1) with FKBP-12 has been described previously to the present invention.

Citation or identification of any reference of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

## *SUMMARY OF THE INVENTION*

The present invention is based, in part, upon the inventors' discovery that p27(Kip1) binds to and forms a complex with a p27(Kip1) binding protein, such as FKBP-12. Accordingly, the present invention discloses herein compositions and methodologies for the production of a protein complex comprised of the p27(Kip1) protein and the protein that interact with (i.e., bind to) said p27(Kip1) protein. Specifically, the invention is directed to a complex of p27(Kip1), or a derivative, fragment or analog thereof, with FKBP-12, or a derivative, analog or fragment thereof. A complex of p27(Kip1) and FKBP-12 is designated as "p27(Kip1)•FKBP-12" herein. The present invention is further directed to methods of screening for proteins that interact with p27(Kip1), or that interact with a derivative, fragment or analog of p27(Kip1). Preferably, the method for screening is a matrix mating test or a variation thereof. See SPECIFIC EXAMPLES, *infra*.

The present invention is also directed to methods for modulating, *i.e.*, inhibiting or enhancing, the activity of a p27(Kip1)•FKBP-12 complex or formation of said complex. The protein components of the p27(Kip1)•FKBP-12 complex have been implicated in a variety of cellular functions, including, but are not limited to, physiological processes such as control of cell cycle progression, cellular differentiation and apoptosis, intracellular signal transduction, neurogenesis, response to viral infection, and pathophysiological processes, which include hyperproliferative disorders such as tumorigenesis and tumor spread, degenerative disorders such as neurodegenerative disease and autoimmune disease, disorders associated with organ transplantation, inflammatory and allergic disease, atherosclerosis, nephropathy and cardiac and muscle disease.

Methods of production of p27(Kip1)•FKBP-12 complex, and derivatives and analogs of the aforementioned protein and protein complex by, for example, recombinant means, are disclosed herein. The present invention further provides methodologies for the modulation (*i.e.*, inhibiting or enhancing) of the activity of the p27(Kip1)•FKBP-12 complex. Accordingly, the present invention provides methodologies for the screening of p27(Kip1)•FKBP-12 complex, as well as derivatives, fragments and analogs thereof, for the ability to modulate or alter cell functions, particularly those cell functions in which p27(Kip1) protein has been implicated including the aforementioned cellular and physiological processes.

Animal models and methodologies of screening for various modulatory agents (*i.e.*, agonists, antagonists and inhibitors) of the activity of p27(Kip1)•FKBP-12 complex are also disclosed herein.

The present invention further relates to therapeutic and prophylactic, as well as diagnostic, prognostic and screening methodologies and pharmaceutical compositions that are based upon p27(Kip1)•FKBP-12 complex (and nucleic acids encoding the individual protein constituents that participate in said complex). Therapeutic compounds of the invention include, but are not limited to, a p27(Kip1)•FKBP-12 complex, or a p27(Kip1)•FKBP-12 complex wherein one or both members of said complex is a derivative, fragment or analog of p27(Kip1) or FKBP-12; antibodies specific to said complex, nucleic acids encoding the foregoing components of said complex, and antisense nucleic acids complementary to the nucleotide sequences encoding the various protein complex components. Kits for diagnostic, prognostic and screening use are also provided.

Methodologies for identification of molecules that inhibit, or alternatively, that increase the formation/synthesis of the p27(Kip1)•FKBP-12 complex are also provided by the present invention.

## *BRIEF DESCRIPTION OF THE DRAWINGS*

Figure 1. The nucleotide sequence (GenBank Accession No. U10906) [SEQ ID NO:1] and deduced amino acid sequence [SEQ ID NO:2] of p27(Kip1). The coding sequence beginning at base 127 (amino acid 43), indicated by an arrow, and ending at base 597 (amino acid 198) was used as bait in the assays described in the SPECIFIC EXAMPLES, *infra*.

Figure 2. The nucleotide sequence (GenBank Accession No. X55741) [SEQ ID NO:3] and deduced amino acid sequence [SEQ ID NO:4] of human FKBP-12. The coding sequence from base 109 (amino acid 34), indicated by an arrow, to the stop codon at base 334 identifies the prey sequence.

Figure 3. Demonstration of the specificity of p27(Kip1)•FKBP-12 interaction. The results of the matrix mating test using p27(Kip1) and proteins A1 and B1 as bait are indicated above the columns

and the prey proteins CDK2 (positive control), FKBP-12, TrkA, CYC-B (cyclophilin B), and Vector (vector control; negative control) are indicated to the left of the rows. A positive interaction between bait and prey proteins is indicated as '+', a lack of interaction is designated by '-'.

## *DETAILED DESCRIPTION OF THE INVENTION*

The present invention is based, in part, upon identification of proteins that interact with p27(Kip1) using a modified form of the yeast matrix mating test. At least amino acids 34 to 107 of FKBP-12 were found to form a complex under physiological conditions with at least amino acids 43 to 198 of p27(Kip1) (the complex of p27(Kip1) with FKBP-12 is indicated as " p27(Kip1)•FKBP-12" herein). The p27(Kip1)•FKBP-12 complex, by virtue of the interaction, is implicated in modulating the functional activities of p27(Kip1) and its binding partner. Such functional activities include, but are not limited to, physiological processes such as control of cell cycle progression, cellular differentiation and apoptosis, intracellular signal transduction, neurogenesis, response to viral infection, and pathophysiological processes including hyperproliferative disorders such as tumorigenesis and tumor spread, degenerative disorders such as neurodegenerative diseases, autoimmune disease, disorders associated with organ transplantation, inflammatory and allergic disease, atherosclerosis, nephropathy and cardiac and muscle diseases.

The present invention is also directed to methods of screening for proteins that interact with, *e.g.*, bind to p27(Kip1). The present invention further discloses a complex of the p27(Kip1) protein, or a derivative, analog or fragment thereof, in particular with FKBP-12 protein, or a derivative, analog or fragment thereof. In a preferred embodiment, such complex binds an anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibody. In another specific embodiment of the present invention, a complex of human p27(Kip1) with human FKBP-12 is provided.

The present invention also provides methodologies for the production and/or isolation of p27(Kip1)•FKBP-12 complex. In a specific embodiment, the present invention provides methodology of using recombinant DNA techniques to express both p27(Kip1) and FKBP-12 (or a derivative, fragment or analog of one or both members of the complex) wherein both binding partners are under the control of one heterologous promoter (*i.e.*, a promoter not naturally associated with the gene encoding the particular complex component) or where each is under the control of a separate heterologous promoter.

Methods of diagnosis, prognosis, and screening for diseases and disorders associated with aberrant levels of a p27(Kip1)•FKBP-12 complex are discloses. The present invention also provides methodology for the treatment or prevention of diseases or disorders associated with an aberrant level of p27(Kip1)•FKBP-12 complex, or an aberrant level of activity of one or more of the components of the complex, by administration of a p27(Kip1)•FKBP-12 complex, or modulators of p27(Kip1)•FKBP-12

complex activity or formation (*e.g.*, antibodies that bind to a p27(Kip1)•FKBP-12 complex), or non-complexed p27(Kip1) or its binding partner or a fragment thereof. Preferably, the aforementioned fragment contains: (i) the portion of p27(Kip1) or FKBP-12 that is directly involved in complex formation; (ii) mutants of p27(Kip1) or of FKBP-12 that modulate binding affinity; (iii) small molecule inhibitors or enhancers of complex formation; or (iv) antibodies that either stabilize or neutralize the complex, and the like.

Methodologies of assaying p27(Kip1)•FKBP-12 complex for biological activity as a therapeutic or diagnostic, as well as methodologies for screening for p27(Kip1)•FKBP-12 complex or modulators thereof (*i.e.*, agonists and antagonists), are also disclosed herein.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections.

**(1) The p27(Kip1) protein, the FKBP-12 protein and the p27(Kip1)•FKBP-12 complex**

The present invention discloses the complex of p27(Kip1) with FKBP-12 (the p27(Kip1)•FKBP-12 complex). In a preferred embodiment, the p27(Kip1)•FKBP-12 complex is a complex of human proteins. The present invention also relates to: (*i*) complexes of derivatives, fragments and analogs of the p27(Kip1) with a FKBP-12; (*ii*) complexes of the p27(Kip1) with derivatives, fragments and analogs of FKBP-12 and (*iii*) complexes of derivatives, fragments and analogs of the p27(Kip1) and FKBP-12. It should be noted that, as used herein, fragment, derivative or analog of a p27(Kip1)•FKBP-12 complex includes complexes where one or both members of the complex are fragments, derivatives or analogs of the wild-type p27(Kip1) or FKBP-12 protein.

Derivatives, fragments, and analogs provided herein are defined as sequences of at least 6 (contiguous) nucleic acids or at least 4 (contiguous) amino acids, a length sufficient to allow for specific hybridization in the case of nucleic acids or for specific recognition of an epitope in the case of amino acids, respectively. Fragments are, at most, one nucleic acid-less or one amino acid-less than the wild type full length sequence. Derivatives and analogs may be full length or other than full length, if said derivative or analog contains a modified nucleic acid or amino acid, as described *infra*. Derivatives or analogs of p27(Kip1) and FKBP-12 include, but are not limited to, molecules comprising regions that are substantially homologous to p27(Kip1) or FKBP-12, in various embodiments, by at least about 30%, 50%, 70%, 80%, or 95% identity (with a preferred identity of 80-95%) over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, or whose encoding nucleic acid is capable of hybridizing to the complement (*e.g.*, the inverse complement) of a sequence encoding p27(Kip1) or FKBP-12 under stringent (the preferred embodiment), moderately stringent, or low stringent conditions. See *e.g.*

Ausubel, *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, NY, 1993, and *infra*.

Preferably, as disclosed by the present invention, the p27(Kip1)•FKBP-12 complex in which one or both members of the complex are a fragment, derivative or analog of the wild-type protein are functionally active p27(Kip1)•FKBP-12 complex. In particular aspects, the native proteins, derivatives or analogs of the p27(Kip1) and/or FKBP-12 are of animals (*e.g.*, mouse, rat, pig, cow, dog, monkey, frog), insects (*e.g.*, fly), plants or, most preferably, human. As utilized herein, the term "functionally active p27(Kip1)•FKBP-12 complex" refers to species displaying one or more known functional attributes of a full-length p27(Kip1) complexed with full-length FKBP-12 including, but not exclusive to, the control of cell cycle progression, cellular differentiation and apoptosis, intracellular signal transduction, neurogenesis, response to viral infection, a hyperproliferative disorder such as tumorigenesis and tumor spread, a degenerative disorder such as a neurodegenerative disease, autoimmune disease, a disorder associated with organ transplantation, inflammatory and/or allergic disease, atherosclerosis, nephropathy, cardiac disease, muscle disease, or the like.

Specific embodiments of the present invention disclose the p27(Kip1)•FKBP-12 complex comprised of fragments of one or both protein species of the complex. In a preferred embodiment, these aforementioned fragments may consist of, but are not limited to, fragments of FKBP-12 that have been identified as interacting with the p27(Kip1) in an improved, modified yeast two hybrid assay in this invention, *i.e.*, amino acids 34-107 of FKBP-12 as depicted in Figure 2 [SEQ ID NO:4]. In addition, fragments (or proteins comprising fragments) that may lack some or all of the aforementioned regions of either member of the complex, as well as nucleic acids that encode the aforementioned proteins, are also disclosed herein.

The nucleotide sequences encoding human p27(Kip1) and human FKBP-12 are known, (GenBank Accession No. U10906 and GenBank Accession No. X55741, respectively), and are disclosed in Figures 1 and 2, SEQ ID NOS:1 and 3, respectively. Nucleic acids may be obtained by any method known within the art (*e.g.*, by PCR amplification using synthetic primers hybridizable to the 3'- and 5'-termini of the sequence and/or by cloning from a cDNA or genomic library using an oligonucleotide sequence specific for the given gene sequence, or the like).

Homologs (*i.e.*, nucleic acids encoding the aforementioned proteins derived from species other than human) or other related sequences (*e.g.*, paralogs) can also be obtained by low, moderate or high stringency hybridization with all or a portion of the particular human sequence as a probe using methods well known in the art for nucleic acid hybridization and cloning.

In a most preferred embodiment, a nucleic acid sequence that is hybridizable to a nucleic acid sequence (or a complement of the foregoing) encoding p27(Kip1) and/or FKBP-12, or a derivative of the same, under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows: Step 1: Filters containing DNA are pretreated for 8 hours to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Step 2: Filters are hybridized for 48 hours at 65°C in the above prehybridization mixture to which is added 100 µg/ml denatured salmon sperm DNA and 5-20 x 106 cpm of $^{32}$P-labeled probe. Step 3: Filters are washed for 1 hour at 37°C in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 minutes. Step 4: Filters are autoradiographed. Other conditions of high stringency that may be used are well known in the art. See, *e.g.*, Ausubel *et al.* (eds.), 1993, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY, and Kriegler, 1990, GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL, Stockton Press, NY.

In a second embodiment, a nucleic acid sequence that is hybridizable to a nucleic acid sequence (or a complement of the foregoing) encoding p27(Kip1) and/or FKBP-12, or a derivative of either, under conditions of moderate stringency is provided. By way of example and not limitation, procedures using such conditions of moderate stringency are as follows: Step 1: Filters containing DNA are pretreated for 6 hours at 55°C in a solution containing 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA. Step 2: Filters are hybridized for 18-20 hours at 55°C in the same solution with 5-20 x 106 cpm $^{32}$P-labeled probe added. Step 3: Filters are washed at 37°C for 1 hour in a solution containing 2X SSC, 0.1% SDS, then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Step 4: Filters are blotted dry and exposed for autoradiography. Other conditions of moderate stringency that may be used are well-known in the art. See, *e.g.*, Ausubel *et al.* (eds.), 1993, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY, and Kriegler, 1990, GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL, Stockton Press, NY.

In a third embodiment, a nucleic acid that is hybridizable to a p27(Kip1) and/or FKBP-12 nucleic acid sequence or to a nucleic acid sequence encoding a p27(Kip1) and/or FKBP-12 derivative (or a complement of the foregoing), under conditions of low stringency, is provided. By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, *Proc. Natl. Acad. Sci. USA 78*: 6789-6792): Step 1: Filters containing DNA are pretreated for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Step 2: Filters are hybridized for 18-20 hours at 40°C in the same solution with the addition of 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 x 106 cpm $^{32}$P-labeled probe. Step 3: Filters are washed for 1.5 hours at 55°C in a solution containing 2X

SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 hours at 60°C. Step 4: Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency that may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations). See, *e.g.*, Ausubel *et al.* (eds.), 1993, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY, and Kriegler, 1990, GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL, Stockton Press, NY.

The invention also relates to nucleic acids hybridizable to or complementary to the foregoing sequences, in particular the invention provides the inverse complement to nucleic acids hybridizable to the foregoing sequences (*i.e.*, the inverse complement of a nucleic acid strand has the complementary sequence running in reverse orientation to the strand so that the inverse complement would hybridize with little or no mismatches to the nucleic acid strand). In specific aspects, nucleic acid molecules are provided that comprise a sequence complementary to (specifically, are the inverse complement of) at least about 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a p27(Kip1) and/or FKBP-12 gene. Nucleic acid molecules encoding derivatives and analogs of p27(Kip1) and/or FKBP-12 (*supra*), or antisense nucleic acids to the same (see, *e.g.*, *infra*) are additionally provided.

Within nucleotide sequences identified as p27(Kip) interactants via the modified yeast two hybrid assay in this invention, potential open reading frames can be identified using the NCBI BLAST program ORF Finder available to the public. Because all known protein translation products are at least 60 amino acids or longer (Creighton, 1992, PROTEINS, 2nd Ed., W.H. Freeman and Co., New York), only those ORFs potentially encoding a protein of 60 amino acids or more are considered. If an initiation methionine codon (ATG) and a translational stop codon (TGA, TAA, or TAG) are identified, then the boundaries of the protein are defined. Other potential proteins include any open reading frames that extend to the 5' end of the nucleotide sequence, in which case the open reading frame predicts the C-terminal or core portion of a longer protein. Similarly, any open reading frame that extends to the 3' end of the nucleotide sequence predicts the N-terminal portion of a longer protein.

**Recombinant Technologies for obtaining the complex or p27(Kip1) or FKBP-12**

The p27(Kip1) and FKBP-12 protein, either alone or within a complex, may be obtained by methods well-known in the art for protein purification and recombinant protein expression. For recombinant expression of one or more of the proteins, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein may be inserted into an appropriate expression vector (*i.e.*, a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence). In a preferred embodiment, the regulatory elements are heterologous (*i.e.*, not the

native gene promoter). Alternately, the necessary transcriptional and translational signals may also be supplied by the native promoter for the p27(Kip1) or any FKBP-12 genes and/or their flanking regions.

A variety of host-vector systems may be utilized to express the protein coding sequence(s). These include, but are not limited to: (*i*) mammalian cell systems that are infected with vaccinia virus, adenovirus, and the like; (*ii*) insect cell systems infected with baculovirus and the like; (*iii*) yeast containing yeast vectors or (*iv*) bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

In a preferred embodiment, the p27(Kip1)•FKBP-12 complex are obtained by expressing the entire p27(Kip1) coding sequence and a FKBP-12 coding sequence within the same cell, either under the control of the same promoter or two separate promoters. In another embodiment, a derivative, fragment or homolog of the p27(Kip1) and/or a derivative, fragment or homolog of a FKBP-12 are recombinantly expressed. Preferably, the derivative, fragment or homolog of the p27(Kip1) and/or the FKBP-12 protein form a complex with a binding partner that has been identified by a binding assay (*e.g.*, the modified yeast two hybrid system assay) and, more preferably, form a complex that binds to an anti-p27(Kip1), anti-FKBP-12 and/or anti-p27(Kip1)•FKBP-12 complex antibody.

Any of the methodologies known within the relevant prior art regarding the insertion of nucleic acid fragments into a vector may be utilized to construct expression vectors that contain a chimeric gene comprised of the appropriate transcriptional/translational control signals and protein-coding sequences. These methodologies may include, but are not limited to, *in vitro* recombinant DNA and synthetic techniques, as well as *in vivo* recombination techniques (*e.g.*, genetic recombination). The expression of nucleic acid sequences that encode the p27(Kip1) and the FKBP-12 protein, or derivatives, fragments, analogs or homologs thereof, may be regulated by a second nucleic acid sequence such that the genes or fragments thereof are expressed in a host that has been concomitantly transformed with the recombinant DNA molecule(s) of interest. The expression of the specific proteins may be controlled by any promoter/enhancer known in the art including, but not limited to: (*i*) the SV40 early promoter (see *e.g.*, Bernoist & Chambon, 1981. *Nature* 290: 304-310); (*ii*) the promoter contained within the 3'-terminus long terminal repeat of Rous Sarcoma Virus (see *e.g.*, Yamamoto, *et al.*, 1980. *Cell* 22: 787-797); (*iii*) the Herpesvirus thymidine kinase promoter (see *e.g.*, Wagner, *et al.*, 1981. *Proc. Natl. Acad. Sci. USA* 78: 1441-1445); (*iv*) the regulatory sequences of the metallothionein gene (see *e.g.*, Brinster, *et al.*, 1982. *Nature* 296: 39-42); (*v*) prokaryotic expression vectors such as the β-lactamase promoter (see *e.g.*, Villa-Kamaroff, *et al.*, 1978. *Proc. Natl. Acad. Sci. USA* 75: 3727-3731); (*vi*) the *tac* promoter (see *e.g.*, DeBoer, *et al.*, 1983. *Proc. Natl. Acad. Sci. USA* 80: 21-25.

In addition, plant promoter/enhancer sequences within plant expression vectors may also be utilized including, but not limited to: (*i*) the nopaline synthetase promoter (see *e.g.,* Herrar-Estrella, *et al.,* 1984. *Nature* 303: 209-213); (*ii*) the cauliflower mosaic virus 35S RNA promoter (see *e.g.,* Garder, *et al.,* 1981. *Nuc. Acids Res.* 9: 2871) and (*iii*) the promoter of the photosynthetic enzyme ribulose bisphosphate carboxylase (see *e.g.,* Herrera-Estrella, *et al.,* 1984. *Nature* 310: 115-120).

Promoter/enhancer elements from yeast and other fungi (*e.g.,* the Gal4 promoter, the alcohol dehydrogenase promoter, the phosphoglycerol kinase promoter, the alkaline phosphatase promoter), as well as from animal transcriptional control regions, for example, those that possess tissue specificity and have been used in transgenic animals, may be utilized in the production of proteins of the present invention. Transcriptional control sequences derived from animals include, but are not limited to: (*i*) the insulin gene control region active within pancreatic β-cells (see *e.g.,* Hanahan, *et al.,* 1985. *Nature* 315: 115-122); (*ii*) the immunoglobulin gene control region active within lymphoid cells (see *e.g.,* Grosschedl, *et al.,* 1984. *Cell* 38: 647-658); (*iii*) the albumin gene control region active within liver (see *e.g.,* Pinckert, *et al.,* 1987. *Genes and Devel.* 1: 268-276; (iv) the myelin basic protein gene control region active within brain oligodendrocyte cells (see *e.g.,* Readhead, *et al.,* 1987. *Cell* 48: 703-712); and (*v*) the gonadotrophin-releasing hormone gene control region active within the hypothalamus (see *e.g.,* Mason, *et al.,* 1986. *Science* 234: 1372-1378), and the like.

In a specific embodiment of the present invention, a vector is utilized that comprises a promoter that is operably-linked to nucleic acid sequences that encode p27(Kip1) and/or FKBP-12, or a fragment, derivative or homolog, thereof, one or more origins of replication, and optionally, one or more selectable markers (*e.g.,* an antibiotic resistance gene). In a preferred embodiment, a vector is utilized that is comprised of a promoter operably-linked to nucleic acid sequences encoding both p27(Kip1) and FKBP-12, one or more origins of replication, and, optionally, one or more selectable markers.

In another specific embodiment, an expression vector contains the coding sequences (or portions thereof) of p27(Kip1) and FKBP-12, either together or separately. The expression vector may be generated by subcloning the aforementioned gene sequences into the *EcoRI* restriction site of each of the three available pGEX vectors (glutathione S-transferase expression vectors; see *e.g.,* Smith & Johnson, 1988. *Gene* 7: 31-40), thus allowing the expression of products in the correct reading frame.

Expression vectors that contain sequences of interest may be identified by three general approaches: (*i*) nucleic acid hybridization, (*ii*) presence or absence of "marker" gene function and/or (*iii*) expression of the inserted sequences. In a first approach, p27(Kip1) and FKBP-12 may be detected by nucleic acid hybridization using probes comprising sequences homologous and complementary to the inserted sequences of interest. In a second approach, the recombinant vector/host system may be identified and selected based upon the presence or absence of certain "marker" functions (*e.g.,* binding to

an antibody specific for t p27(Kip1), FKBP-12, or a p27(Kip1)•FKBP-12 complex, resistance to antibiotics, occlusion-body formation in baculovirus, and the like) caused by the insertion of the sequences of interest into the vector. In a third approach, recombinant expression vectors may be identified by assaying for the expression of the p27(Kip1) concomitantly with expression of FKBP-12 by the recombinant vector.

Once the recombinant p27(Kip1) and FKBP-12 molecules have been identified and the complex or individual proteins isolated, and a suitable host system and growth conditions have been established, the recombinant expression vectors may be propagated and amplified in-quantity. As previously discussed, expression vectors or their derivatives that can be used include, but are not limited to, human or animal viruses (*e.g.*, vaccinia virus or adenovirus); insect viruses (*e.g.*, baculovirus); yeast vectors; bacteriophage vectors (*e.g.*, lambda phage); plasmid vectors and cosmid vectors.

A host cell strain may be selected that modulates the expression of inserted sequences of interest, or modifies or processes expressed proteins encoded by said sequences in the specific manner desired. In addition, expression from certain promoters may be enhanced in the presence of certain inducers in a selected host strain; thus facilitating control of the expression of a genetically-engineered p27(Kip1) and/or FKBP-12. Moreover, different host cells possess characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation, and the like) of expressed proteins. Appropriate cell lines or host systems may thus be chosen to ensure the desired modification and processing of the foreign protein is achieved. For example, protein expression within a bacterial system can be used to produce an unglycosylated core protein; whereas expression within mammalian cells ensures "native" glycosylation of a heterologous protein.

In other specific embodiments, p27(Kip1) and/or FKBP-12 (or derivatives, fragments, analogs and homologs thereof) may be expressed as fusion or chimeric protein products comprising the protein joined via a peptide bond to a heterologous protein sequence of a different protein. Such chimeric products may be produced by ligating together appropriate nucleic acid sequences that encode desired amino acids, said ligation retaining the proper coding frames, and subsequently expressing the chimeric products in a suitable host by methods well known in the art. Alternatively, such a chimeric product can be made by protein synthetic techniques (*e.g.*, by use of a peptide synthesizer).

A specific embodiment of the present invention discloses a chimeric protein comprising a fragment of p27(Kip1) and/or FKBP-12. In another specific embodiment, fusion proteins are provided that contain the interacting domains of p27(Kip1) and FKBP-12 (the domains involved in the direct formation of p27(Kip1)•FKBP-12 complex) and, optionally, have a heterofunctional reagent (*e.g.*, a peptide linker) that serves to both link the two aforementioned proteins and to promote the interaction of p27(Kip1) and FKBP-12 binding domains. These fusion proteins may be particularly useful where the

stability of the interaction is desirable (*i.e.*, stability due to the formation of the complex as an intramolecular reaction), for example in production of antibodies specific to p27(Kip1)•FKBP-12 complex.

In a specific embodiment of the present invention, the nucleic acids encoding proteins, and proteins consisting of or comprising a fragment of p27(Kip1) or FKBP-12 that consists of a minimum of 6 contiguous amino acid residues of p27(Kip1) and/or FKBP-12, are provided herein. In another embodiment, the aforementioned protein fragment is comprised of at least 10, 20, 30, 40, or 50 amino acid residues (and preferably not larger that 35, 100 or 200 amino acid residues) of p27(Kip1) or FKBP-12. Derivatives or analogs of p27(Kip1) and FKBP-12 include, but are not limited to, molecules comprising regions that are substantially homologous to p27(Kip1) or FKBP-12 in various embodiments, of at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or preferably 95% amino acid identity when: (*i*) compared to an amino acid sequence of identical size; (*ii*) compared to an aligned sequence in that the alignment is done by a computer homology program known within the art or (*iii*) the encoding nucleic acid is capable of hybridizing to a sequence encoding p27(Kip1) or FKBP-12 under stringent (preferred), moderately stringent, or non-stringent conditions (see, *e.g.*, *supra*).

p27(Kip1) and/or FKBP-12 derivatives may be produced by alteration of their sequences by substitutions, additions or deletions that result in functionally-equivalent molecules. In a specific embodiment of the present invention, the degeneracy of nucleotide coding sequences allows for the use of other DNA sequences that encode substantially the same amino acid sequence as p27(Kip1) or FKBP-12 genes. In another specific embodiment, one or more amino acid residues within the sequence of interest may be substituted by another amino acid of a similar polarity and net charge, thus resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. Positively charged (basic) amino acids include arginine, lysine and histidine. Negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

p27(Kip1) or FKBP-12 derivatives and analogs of the present invention may be produced by various methodologies known within the art. For example, the cloned p27(Kip1) and FKBP-12 gene sequences may be modified by any of numerous methods known within the art. See *e.g.*, Sambrook, *et al.*, 1990. *Molecular Cloning: A Laboratory Manual, 2nd ed.*, (Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY). These sequences may be digested at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification, if so desired, and the resultant fragments isolated and ligated *in vitro*. Additionally, p27(Kip1)- or FKBP-12-encoding nucleic acids may be

mutated *in vitro* or *in vivo* to: (*i*) create variations in coding regions; (*ii*) create and/or destroy translation, initiation, and/or termination sequences; and/or (*iii*) form new restriction endonuclease sites or destroy pre-existing ones, so as to facilitate further *in vitro* modification. Any technique for mutagenesis known within the art may be utilized, including but not limited to, chemical mutagenesis and *in vitro* site-directed mutagenesis (see *e.g.*, Hutchinson, *et al.*, 1978. *J. Biol. Chem 253*: 6551-6558); use of TABJ™ linkers (Pharmacia), and other similar methodologies.

**Isolation and analysis of the gene product or complex**

Once a recombinant cell expressing p27(Kip1) and/or FKBP-12, or a fragment or derivative thereof, is identified, the individual gene product or complex may be isolated and analyzed. This is achieved by assays that are based upon the physical and/or functional properties of the protein or complex, including, but not limited to, radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, cross-linking to marker-labeled products, and the like. The p27(Kip1)•FKBP-12 complex may be isolated and purified by standard methods known in the art (either from natural sources or recombinant host cells expressing the proteins/protein complex) including, but not limited to, column chromatography (*e.g.*, ion exchange, affinity, gel exclusion, reverse-phase, high pressure, fast protein liquid, etc), differential centrifugation, differential solubility, or similar methodologies used for the purification of proteins. Alternatively, once p27(Kip1) or FKBP-12 or its derivative is identified, the amino acid sequence of the protein can be deduced from the nucleic acid sequence of the chimeric gene from which it was encoded. Hence, the protein or its derivative can be synthesized by standard chemical methodologies known in the art. See, *e.g.*, Hunkapiller, *et al.*, 1984. *Nature* 310: 105-111.

In a specific embodiment, a p27(Kip1)•FKBP-12 complex (whether produced by recombinant DNA techniques, chemical synthesis methods, or by purification from native sources) is made up from proteins, fragments, analogs and derivatives thereof, that, as their primary amino acid, contain sequences substantially as depicted in Figures 1 and 2, as well as proteins homologous thereto.

**Manipulations of the p27(KIP1) and/or FKBP-12 sequences**

Manipulations of the p27(Kip1) and/or FKBP-12 sequences may be made at the protein level. Included within the scope of the present invention are complex of the p27(Kip1) or FKBP-12 fragments, derivatives, fragments or analogs that are differentially modified during or after translation (*e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, and the like). Any of the numerous chemical modification methodologies known within the art may be utilized including,

but not limited to, specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc. In a specific embodiment, the p27(Kip1) and/or FKBP-12 sequences are modified to include a fluorescent label. In another specific embodiment, the p27(Kip1) and/or the FKBP-12 are modified by the incorporation of a heterofunctional reagent, wherein such heterofunctional reagent may be used to cross-link the members of the complex.

**Chemical synthesis**

Complexes of analogs and derivatives of p27(Kip1) and/or FKBP-12 can be chemically synthesized. For example, a peptide corresponding to a portion of p27(Kip1) and/or FKBP-12 that comprises the desired domain or that mediates the desired activity *in vitro* (*e.g.*, p27(Kip1)•FKBP-12 complex formation), may be synthesized by use of a peptide synthesizer. In cases where natural products are suspected of being mutant or are isolated from new species, the amino acid sequence of p27(Kip1) and/or FKBP-12 isolated from the natural source, as well as those expressed *in vitro*, or from synthesized expression vectors *in vivo* or *in vitro*, may be determined from analysis of the DNA sequence, or alternatively, by direct sequencing of the isolated protein. The p27(Kip1)•FKBP-12 complex may also be analyzed by hydrophilicity analysis (see *e.g.*, Hopp & Woods, 1981. *Proc. Natl. Acad. Sci. USA* 78: 3824-3828) that can be utilized to identify the hydrophobic and hydrophilic regions of the proteins, thus aiding in the design of substrates for experimental manipulation, such as in binding experiments, antibody synthesis, etc. Secondary structural analysis may also be performed to identify regions of the p27(Kip1) and/or FKBP-12 that assume specific structural motifs. See *e.g.*, Chou & Fasman, 1974. *Biochem.* 13: 222-223. Manipulation, translation, secondary structure prediction, hydrophilicity and hydrophobicity profiles, open reading frame prediction and plotting, and determination of sequence homologies, can be accomplished using computer software programs available in the art. Other methods of structural analysis including, but not limited to, X-ray crystallography (see *e.g.*, Engstrom, 1974. *Biochem. Exp. Biol.* 11: 7-13); mass spectroscopy and gas chromatography (see *e.g.*, METHODS IN PROTEIN SCIENCE, 1997. J. Wiley and Sons, New York, NY) and computer modeling (see *e.g.*, Fletterick & Zoller, eds., 1986. Computer Graphics and Molecular Modeling, In: CURRENT COMMUNICATIONS IN MOLECULAR BIOLOGY, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) may also be employed.

**Methodologies for screening**

The present invention provides methodologies for screening p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complexes, as well as derivatives, fragments and analogs thereof, for the ability to alter and/or modulate cellular functions, particularly those functions in which p27(Kip1) and/or

FKBP-12 have been implicated. These functions include, but are not limited to, control of cell-cycle progression; regulation of transcription; control of intracellular signal transduction; and pathological processes, as well as various other biological activities (*e.g.*, binding to an anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibody, and the like). The derivatives, fragments or analogs that possess the desired immunogenicity and/or antigenicity may be utilized in immunoassays, for immunization, for inhibition of p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity, etc. For example, derivatives, fragments or analogs that retain, or alternatively lack or inhibit, a given property of interest (*e.g.*, participation in a p27(Kip1)•FKBP-12 complex) may be utilized as inducers, or inhibitors, respectively, of such a property and its physiological correlates. In a specific embodiment, a p27(Kip1)•FKBP-12 complex of a fragment of the p27(Kip1) and/or a fragment of FKBP-12 that can be bound by an anti-p27(Kip1) and/or anti-FKBP-12 antibody or antibody specific for a p27(Kip1)•FKBP-12 complex when such a fragment is included within a given p27(Kip1)•FKBP-12 complex. Derivatives, fragments and analogs of p27(Kip1)•FKBP-12 complex may be analyzed for the desired activity or activities by procedures known within the art.

**(2) Production of antibodies to the p27(Kip1)•FKBP-12 complex**

As disclosed by the present invention herein, p27(Kip1)•FKBP-12 complex, or derivatives, fragments, analogs or homologs thereof, may be utilized as immunogens in the generation of antibodies that immunospecifically-bind these protein components. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, $F_{ab}$ fragments and an $F_{ab}$ expression library. In a specific embodiment, antibodies to a complex of human p27(Kip1) and human FKBP-12 are disclosed. In another specific embodiment, complex formed from fragments of p27(Kip1) and FKBP-12; wherein these fragments contain the protein domain that interacts with the other member of the complex and are used as immunogens for antibody production. Various procedures known within the art may be used for the production of polyclonal or monoclonal antibodies to a p27(Kip1)•FKBP-12 complex, or derivative, fragment, analog or homolog thereof.

For the production of polyclonal antibodies, various host animals may be immunized by injection with the native p27(Kip1)•FKBP-12 complex, or a synthetic variant thereof, or a derivative of the foregoing (*e.g.*, a cross-linked p27(Kip1)•FKBP-12). Various adjuvants may be used to increase the immunological response and include, but are not limited to, Freund's (complete and incomplete), mineral gels (*e.g.*, aluminum hydroxide), surface active substances (*e.g.*, lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, etc.) and human adjuvants such as *Bacille Calmette-Guerin* and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed towards a p27(Kip1)•FKBP-12 complex, or derivatives, fragments, analogs or homologs thereof, any technique that provides for the production of antibody molecules by continuous cell line culture may be utilized. Such techniques include, but are not limited to, the hybridoma technique (see Kohler & Milstein, 1975. *Nature* 256: 495-497); the trioma technique; the human B-cell hybridoma technique (see Kozbor, *et al.*, 1983. *Immunol. Today* 4: 72) and the EBV hybridoma technique to produce human monoclonal antibodies (see Cole, *et al.*, 1985. In: *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Human monoclonal antibodies may be utilized in the practice of the present invention and may be produced by the use of human hybridomas (see Cote, *et al.*, 1983. *Proc. Natl. Acad. Sci. USA* 80: 2026-2030) or by transforming human B-cells with Epstein Barr Virus *in vitro* (see Cole, *et al.*, 1985. In: *Monoclonal Antibodies and Cancer Therapy* (Alan R. Liss, Inc., pp. 77-96).

According to the invention, techniques can be adapted for the production of single-chain antibodies specific to p27(Kip1)•FKBP-12 complex (see *e.g.*, U.S. Patent No. 4,946,778). In addition, methodologies can be adapted for the construction of $F_{ab}$ expression libraries (see *e.g.*, Huse, *et al.*, 1989. *Science* 246: 1275-1281) to allow rapid and effective identification of monoclonal $F_{ab}$ fragments with the desired specificity for p27(Kip1)•FKBP-12 or derivatives, fragments, analogs or homologs thereof. Non-human antibodies can be "humanized" by techniques well known in the art. See *e.g.*, U.S. Patent No. 5,225,539. Antibody fragments that contain the idiotypes to p27(Kip1)•FKBP-12 complex may be produced by techniques known in the art including, but not limited to: (*i*) an $F_{(ab')2}$ fragment produced by pepsin digestion of an antibody molecule; (*ii*) an $F_{ab}$ fragment generated by reducing the disulfide bridges of an $F_{(ab')2}$ fragment; (*iii*) an $F_{ab}$ fragment generated by the treatment of the antibody molecule with papain and a reducing agent and (*iv*) $F_v$ fragments.

In one embodiment, methodologies for the screening of antibodies that possess the desired specificity include, but are not limited to, enzyme-linked immunosorbent assay (ELISA) and other immunologically-mediated techniques known within the art. In a specific embodiment, selection of antibodies that are specific to a particular domain of p27(Kip1)•FKBP-12 complex is facilitated by generation of hybridomas that bind to the fragment of p27(Kip1)•FKBP-12 complex possessing such a domain. In another specific embodiment, methodologies for the selection of an antibody that specifically binds a p27(Kip1)•FKBP-12 complex but that does not specifically bind to the individual proteins of p27(Kip1)•FKBP-12 complex (identified by selecting the antibody on the basis of positive-binding to p27(Kip1)•FKBP-12 complex with a concomitant lack of binding to the individual p27(Kip1) and FKBP-12 protein) are within the scope of the invention. Accordingly, antibodies that are specific for a domain within p27(Kip1)•FKBP-12 complex, or derivative, fragments, analogs or homologs thereof, are also provided herein.

It should be noted that the aforementioned antibodies may be used in methods known within the art relating to the localization and/or quantitation of p27(Kip1)•FKBP-12 complex (*e.g.*, for use in measuring levels of the protein within appropriate physiological samples, for use in diagnostic methods, for use in imaging the protein, and the like). In a given embodiment, anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibodies, or derivatives, fragments, analogs or homologs thereof that contain the antibody derived binding domain, are utilized as pharmacologically-active compounds [hereinafter "Therapeutics"].

**(3) Use of p27(Kip1)•FKBP-12 Complex in Diagnosis, Prognosis and Screening**

p27(Kip1)•FKBP-12 complex may serve as a "marker" for specific disease states that involve the disruption of physiological processes in which p27(Kip1) and FKBP-12 are known to be involved. See, *e.g.*, BACKGROUND OF THE INVENTION. These physiological processes include, but are not limited to, (i) control of cell cycle progression, cellular differentiation and apoptosis, (ii) intracellular signal transduction, (iii) neurogenesis, (iv) response to viral infection; and (v) pathophysiological processes including, but not limited to, hyperproliferative disorders such as tumorigenesis and tumor spread, degenerative disorders such as neurodegenerative diseases, autoimmune diseases, disorders associated with organ transplantation, inflammatory and allergic diseases, atherosclerosis, nephropathy and cardiac muscle diseases, and the like. Thus p27(Kip1)•FKBP-12 complexes are predicted to have diagnostic utility. Therefore, the differentiation and classification of particular groups of patients possessing elevations or deficiencies of a p27(Kip1)•FKBP-12 complex may lead to new nosological classifications of diseases, thereby markedly advancing diagnostic ability.

The detection of levels of p27(Kip1)•FKBP-12 complex or levels of p27(Kip1) and/or FKBP-12 protein, or detection of levels of mRNAs that encode the components of a p27(Kip1)•FKBP-12 complex, may be utilized in the analysis of various diseases, and may provide critical information in various medical processes, including: diagnosis, prognosis, identifying disease states, following a disease course, following the efficacy of an administered therapeutics, following therapeutic response, and the like. Similarly, both the nucleic acid sequences (and sequences complementary thereto) and antibodies specific to p27(Kip1)•FKBP-12 complex and/or the individual components that can form p27(Kip1)•FKBP-12 complexes, can be used in diagnostics.

Said molecules may be utilized in assays (*e.g.*, immunoassays) to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders characterized by aberrant levels of p27(Kip1)•FKBP-12 complex, or monitor the treatment thereof. An "aberrant level" means an increased or decreased level in a sample relative to that present in an analogous sample from an unaffected part of the body, or from a subject not having the disorder. The aforementioned immunoassay may be

performed by a methodology comprising contacting a sample derived from a patient with an anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibody under conditions such that immunospecific-binding may occur, and subsequently detecting or measuring the amount of any immunospecific-binding by the antibody. In a specific embodiment, an antibody specific for p27(Kip1), FKBP-12, and/or a p27(Kip1)•FKBP-12 complex may be used to analyze a tissue or serum sample from a patient for the presence of uncomplexed or complexed p27(Kip1)•FKBP-12; wherein an aberrant level of p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex is indicative of a diseased condition. The immunoassays that may be utilized include, but are not limited to, competitive and non-competitive assay systems using techniques such as Western Blots, radioimmunoassays (RIA), enzyme linked immunosorbent assay (ELISA), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein-A immunoassays, etc.

The nucleic acid species of the present invention encoding the associated protein components of p27(Kip1)•FKBP-12 complex, and related nucleotide sequences and subsequences, may also be used in hybridization assays. p27(Kip1) and FKBP-12 nucleotide sequences, or subsequences thereof comprising at least 6 nucleotides, may be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant levels of the mRNAs encoding the components of a p27(Kip1)•FKBP-12 complex, as described *supra*. In specific embodiments of the present invention, diseases and disorders involving or characterized by aberrant levels of p27(Kip1)•FKBP-12 complex or a predisposition to develop such disorders may be diagnosed by detecting aberrant levels of p27(Kip1)•FKBP-12 complex, or non-complexed p27(Kip1) and/or FKBP-12 proteins or nucleic acids for functional activity. This aforementioned functional activity may include, but is not restricted to, (*i*) binding to an interacting partner (*e.g.*, p27(Kip1), FKBP-12) or (*ii*) detecting mutations in p27(Kip1) and/or a FKBP-12 RNA, DNA or protein (*e.g.*, translocations, truncations, changes in nucleotide or amino acid sequence relative to wild-type p27(Kip1) and/or the FKBP-12) that can cause increased or decreased expression or activity of a p27(Kip1), a FKBP-12 or a p27(Kip1)•FKBP-12 complex.

Methodologies that are well-known within the art (*e.g.*, immunoassays, nucleic acid hybridization assays, biological activity assays, and the like) may be used to determine whether one or more particular p27(Kip1)•FKBP-12 complexes are present at either increased or decreased levels, or are absent, within samples derived from patients suffering from a particular disease or disorder, or possessing a predisposition to develop such a disease or disorder, as compared to the levels in samples from subjects not having such disease or disorder or predisposition thereto. Additionally, these assays may be utilized to determine whether the ratio of p27(Kip1)•FKBP-12 complex to the non-complexed

components (*i.e.* p27(Kip1) and/or FKBP-12) in the complex of interest is increased or decreased in samples from patients suffering from a particular disease or disorder or having a predisposition to develop such a disease or disorder as compared to the ratio in samples from subjects not having such a disease or disorder or predisposition thereto.

Accordingly, in specific embodiments of the present invention, diseases and disorders that involve increased/decreased levels of one or more p27(Kip1)•FKBP-12 complex may be diagnosed, or their suspected presence may be screened for, or a predisposition to develop such diseases and disorders may be detected, by quantitatively ascertaining increased/decreased levels of: (*i*) the one or more p27(Kip1)•FKBP-12 complex ; (*ii*) the mRNA encoding both protein members of said complex; (*iii*) the complex functional activity or (*iv*) mutations in p27(Kip1) or the FKBP-12 (*e.g.*, translocations in nucleic acids, truncations in the gene or protein, changes in nucleotide or amino acid sequence relative to wild-type p27(Kip1) or the FKBP-12) that enhance/inhibit or stabilize/destabilize p27(Kip1)•FKBP-12 complex formation.

In the practice of the present invention, the use of detection techniques, especially those involving antibodies directed against p27(Kip1)•FKBP-12 complex, provide methods for the detection of specific cells that express the uncomplexed or complexed protein of interest, *e.g.*, p27(Kip1) and/or FKBP-12 . Using such assays, specific cell types may be quantitatively characterized in which one or more particular components of a p27(Kip1)•FKBP-12 complex are expressed, and the presence of the uncomplexed or complexed protein may be correlated with cell viability by techniques well-known within the art (*e.g.*, fluorescence-activated cell sorting). Also embodied herein are methodologies directed to the detection of a p27(Kip1)•FKBP-12 complex within *in vitro* cell culture models that express a particular p27(Kip1)•FKBP-12 complex, or derivatives thereof, for the purpose of characterizing and/or isolating p27(Kip1)•FKBP-12 complex. These detection techniques include, but are not limited to, cell-sorting of prokaryotes (see *e.g.*, Davey & Kell, 1996. *Microbiol. Rev.* 60: 641-696); primary cultures and tissue specimens from eukaryotes, including mammalian species such as human (see *e.g.*, Steele, *et al.*, 1996. *Clin. Obstet. Gynecol.* 39: 801-813) and continuous cell cultures (see *e.g.*, Orfao & Ruiz-Arguelles, 1996. *Clin. Biochem.* 29: 5-9.

The present invention additionally provides kits for diagnostic use that are comprised of one or more containers containing an anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibody and, optionally, a labeled binding partner to said antibody. The label incorporated into the anti-p27(Kip1)•FKBP-12 complex antibody may include, but is not limited to, a chemiluminescent, enzymatic, fluorescent, colorimetric or radioactive moiety. In another specific embodiment, kits for diagnostic use that are comprised of one or more containers containing modified or unmodified nucleic acids that encode, or alternatively, that are the complement to, p27(Kip1), FKBP-12, and/or

p27(Kip1)•FKBP-12 complex and, optionally, a labeled binding partner to said nucleic acids, are also provided. In an alternative specific embodiment, the kit may comprise, in one or more containers, a pair of oligonucleotide primers (*e.g.*, each 6-30 nucleotides in length) that are capable of acting as amplification primers for polymerase chain reaction (PCR; see *e.g.*, Innis, *et al.*, 1990. PCR PROTOCOLS, Academic Press, Inc., San Diego, CA), ligase chain reaction, cyclic probe reaction, and the like, or other methods known within the art. The kit may, optionally, further comprise a predetermined amount of a purified p27(Kip1), FKBP-12 or p27(KIP1)•FKBP-12 complex, or nucleic acids thereof, for use as a diagnostic, standard, or control in the aforementioned assays.

**(4) Therapeutic uses of p27(Kip1) and FKBP-12 proteins and p27(Kip1) •FKBP-12 complexes**

The present invention provides a method for treatment or prevention of various diseases and disorders by administration of a biologically-active therapeutic compound (hereinafter "Therapeutic"). Such "Therapeutics" include but are not limited to: (i) p27(Kip1), FKBP-12, and p27(Kip1)•FKBP-12 complex, and derivative, fragments, analogs and homologs thereof; (ii) antibodies directed against the aforementioned proteins and protein complex thereof; (iii) nucleic acids encoding p27(Kip1) and/or FKBP-12, and derivatives, fragments, analogs and homologs thereof; (iv) antisense nucleic acids to sequences encoding p27(Kip1) and FKBP-12 proteins, and (v) p27(Kip1)•FKBP-12 complex and modulators thereof ( *i.e.*, inhibitors, agonists and antagonists).

As previously discussed, p27(Kip1) and its binding partner FKBP-12, are implicated significantly in disorders of cell cycle progression and cell differentiation, including cancer and tumorigenesis and tumor progression. Disorders of neurodegeneration resulting from altered cellular apoptosis, differentiation, and DNA repair likewise involves these same proteins. A wide range of cell diseases affected by physiological processes such as control of cell cycle progression, cellular differentiation and apoptosis, intracellular signal transduction, neurogenesis, response to viral infection; and pathophysiological processes including but not limited to hyperproliferative disorders such as tumorigenesis and tumor spread, degenerative disorders such as neurodegenerative diseases, disorders associated with organ transplantation, inflammatory and allergic diseases, autoimmune diseases, atherosclerosis, nephropathy, and cardiac and muscle diseases are treated or prevented by administration of a Therapeutic that modulates, *i.e.*, antagonizes or promotes, p27(Kip1)•FKBP-12 complex activity or formation.

Diseases or disorders associated with aberrant levels of a p27(Kip1)•FKBP-12 complex or levels of activity or aberrant levels of p27(Kip1) may be treated by administration of a Therapeutic that modulates p27(Kip1)•FKBP-12 complex formation or activity. In a specific embodiment, the activity or

levels of p27(Kip1) are modulated by administration of FKBP-12. In another specific embodiment, the activity or levels of FKBP-12 are modulated by administration of p27(Kip1).

**Disorders with Increased p27(Kip1) and p27(Kip1)•FKBP-12 Complex Levels**

Diseases and disorders that are characterized by increased (relative to a subject not suffering from said disease or disorder) p27(Kip1)•FKBP-12 levels or biological activity may be treated with Therapeutics that antagonize (*i.e.*, reduce or inhibit) p27(Kip1)•FKBP-12 complex formation or activity. Therapeutics that antagonize p27(Kip1)•FKBP-12 complex formation or activity may be administered in a therapeutic or prophylactic manner. Therapeutics that may be utilized include, but are not limited to, p27(Kip1) or FKBP-12, or analogs, derivatives, fragments or homologs thereof; (*ii*) anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibodies; (*iii*) nucleic acids encoding p27(Kip1) or FKBP-12; (*iv*) concurrent administration of a p27(Kip1) and a FKBP-12 antisense nucleic acid and p27(Kip1) and/or FKBP-12 nucleic acids that are "dysfunctional" (*i.e.*, due to a heterologous [non-p27(Kip1) and/or non-FKBP-12] insertion within the coding sequences of p27(Kip1) and FKBP-12 coding sequences) are utilized to "knockout" endogenous p27(Kip1) and/or FKBP-12 function by homologous recombination (see *e.g.*, Capecchi, 1989. *Science* 244: 1288-1292). In an additionally embodiment of the present invention, mutants or derivatives of a first FKBP-12 that possess greater affinity for p27(Kip1) than the wild-type first FKBP-12 may be administered to compete with a second FKBP-12 for binding to p27(Kip1), thereby reducing the levels of complex between p27(Kip1) and the second FKBP-12.

Increased levels of p27(Kip1)•FKBP-12 complex can be readily detected by quantifying protein and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or protein levels, structure and/or activity of the expressed p27(Kip1)•FKBP-12 complex (or p27(Kip1) and FKBP-12 mRNAs). Methods that are well-known within the art include, but are not limited to, immunoassays to detect p27(Kip1)•FKBP-12 complex (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect concurrent expression of p27(Kip1) and FKBP-12 mRNAs (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.).

**Reduction of p27(Kip1) and p27(Kip1)•FKBP-12 Complex Expression**

A specific embodiment of the present invention discloses methods for the reduction of p27(Kip1)•FKBP-12 complex expression (*i.e.*, the expression of the two protein components of the complex and/or formation of the complex) by targeting mRNAs that express the protein moieties. RNA Therapeutics are differentiated into three classes: (*i*) antisense species; (*ii*) ribozymes or (*iii*) RNA

aptamers. See *e.g.*, Good, *et al.*, 1997. *Gene Therapy* 4: 45-54. Antisense oligonucleotides have been the most widely utilized and are discussed *infra*. Ribozyme therapy involves the administration (*i.e.*, induced expression) of small RNA molecules with enzymatic ability to cleave, bind, or otherwise inactivate specific RNAs, thus reducing or eliminating the expression of particular proteins. See *e.g.*, Grassi & Marini, 1996. *Ann. Med.* 28: 499-510. At present, the design of "hairpin" and/or "hammerhead" RNA ribozymes are necessary to specifically-target a particular mRNA (*e.g.*, p27(Kip1) mRNA). RNA aptamers are specific RNA ligands for proteins, such as for *Tat* and *Rev* RNA (see *e.g.*, Good, *et al.*, 1997. *Gene Therapy* 4: 45-54) which can specifically inhibit their translation.

In a preferred embodiment of the present invention, the activity or level of p27(Kip1) may be reduced by administration of FKBP-12, a nucleic acid that encodes FKBP-12 or an antibody (or a derivative or fragment of the antibody possessing the binding domain thereof) that immunospecifically-binds to FKBP-12. Similarly, the levels or activity of FKBP-12 may be reduced by administration of p27(Kip1), a nucleic acid encoding p27(Kip1) or an antibody (or a derivative or fragment of the antibody possessing the binding domain thereof) that immunospecifically-binds p27(Kip1). In another embodiment of the present invention, diseases or disorders that are associated with increased levels of p27(Kip1) or FKBP-12, may be treated or prevented by administration of a Therapeutic that increases p27(Kip1)•FKBP-12 complex formation, if said complex formation acts to reduce or inactivate p27(Kip1) or the particular FKBP-12 via p27(Kip1)•FKBP-12 complex formation. Such diseases or disorders may be treated or prevented by: (*i*) the administration of one member of p27(Kip1)•FKBP-12 complex, including mutants of one or both of the proteins that possess increased affinity for the other member of p27(Kip1)•FKBP-12 complex (so as to cause increased complex formation) or (*ii*) the administration of antibodies or other molecules that serve to stabilize p27(Kip1)•FKBP-12 complex, or the like.

**(5) Determination of the Biological Effect of the Therapeutic**

In preferred embodiments of the present invention, suitable *in vitro* or *in vivo* assays are utilized to determine the effect of a specific Therapeutic and whether its administration is indicated for treatment of the affected tissue.

In various specific embodiments, *in vitro* assays may be performed with representative cells of the type(s) involved in the patient's disorder, to determine if a given Therapeutic exerts the desired effect upon said cell type(s). Compounds for use in therapy may be tested in suitable animal model systems including, but not limited to rats, mice, chicken, cows, monkeys, rabbits, and the like, prior to testing in human subjects. Similarly, for *in vivo* testing, any of the animal model system known in the art may be used prior to administration to human subjects.

**Malignancies**

Components of p27(Kip1)•FKBP-12 complex are involved in the regulation of cell proliferation. Accordingly, Therapeutics of the present invention may be useful in the therapeutic or prophylactic treatment of diseases or disorders that are associated with cell hyperproliferation and/or loss of control of cell proliferation (*e.g.*, cancers, malignancies and tumors). For a review of such hyperproliferation disorders, see *e.g.*, Fishman, *et al.*, 1985. MEDICINE, 2nd ed., J.B. Lippincott Co., Philadelphia, PA.

Therapeutics of the present invention may be assayed by any method known within the art for efficacy in treating or preventing malignancies and related disorders. Such assays include, but are not limited to, *in vitro* assays utilizing transformed cells or cells derived from the patient's tumor, as well as *in vivo* assays using animal models of cancer or malignancies. Potentially effective Therapeutics are those that, for example, inhibit the proliferation of tumor-derived or transformed cells in culture or cause a regression of tumors in animal models, in comparison to the controls.

In the practice of the present invention, once a malignancy or cancer has been shown to be amenable to treatment by modulating (*i.e.*, inhibiting, antagonizing or agonizing) p27(Kip1)•FKBP-12 complex activity, that cancer or malignancy may subsequently be treated or prevented by the administration of a Therapeutic that serves to modulate p27(Kip1)•FKBP-12 complex formation and function, including supplying p27(Kip1)•FKBP-12 complex and/or the individual binding partners of said protein complex (*i.e.*, p27(Kip1) and/or FKBP-12).

**Premalignant conditions**

The Therapeutics of the present invention that are effective in the therapeutic or prophylactic treatment of cancer or malignancies may also be administered for the treatment of pre-malignant conditions and/or to prevent the progression of a pre-malignancy to a neoplastic or malignant state. Such prophylactic or therapeutic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia or, most particularly, dysplasia has occurred. For a review of such abnormal cell growth see *e.g.*, Robbins & Angell, 1976. BASIC PATHOLOGY, 2nd ed., W.B. Saunders Co., Philadelphia, PA.

Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in its structure or function. For example, it has been demonstrated that endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of mature or fully differentiated cell substitutes for another type of mature cell. Metaplasia may occur in epithelial or connective tissue cells. Dysplasia is generally considered a precursor of cancer, and is found mainly in the epithelia. Dysplasia is the most disorderly

form of non-neoplastic cell growth, and involves a loss in individual cell uniformity and in the architectural orientation of cells. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder.

Alternatively, or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed or malignant phenotype displayed either *in vivo* or *in vitro* within a cell sample derived from a patient, is indicative of the desirability of prophylactic/therapeutic administration of a Therapeutic that possesses the ability to modulate p27(Kip1)•FKBP-12 complex activity. Characteristics of a transformed phenotype include, but are not limited to: (*i*) morphological changes; (*ii*) looser substratum attachment; (*iii*) loss of cell-to-cell contact inhibition; (*iv*) loss of anchorage dependence; (*v*) protease release; (*vi*) increased sugar transport; (*vii*) decreased serum requirement; (*viii*) expression of fetal antigens, (*ix*) disappearance of the 250 kDal cell-surface protein, and the like. See *e.g.*, Richards, *et al.*, 1986. MOLECULAR PATHOLOGY, W.B. Saunders Co., Philadelphia, PA.

In a specific embodiment of the present invention, a patient that exhibits one or more of the following predisposing factors for malignancy is treated by administration of an effective amount of a Therapeutic: (*i*) a chromosomal translocation associated with a malignancy (*e.g.*, the Philadelphia chromosome (*bcr/abl*) for chronic myelogenous leukemia and t(14;18) for follicular lymphoma, etc.); (*ii*) familial polyposis or Gardner's syndrome (possible forerunners of colon cancer); (*iii*) monoclonal gammopathy of undetermined significance (a possible precursor of multiple myeloma) and (*iv*) a first degree kinship with persons having a cancer or pre-cancerous disease showing a Mendelian (genetic) inheritance pattern (*e.g.*, familial polyposis of the colon, Gardner's syndrome, hereditary exostosis, polyendocrine adenomatosis, Peutz-Jeghers syndrome, neurofibromatosis of Von Recklinghausen, medullary thyroid carcinoma with amyloid production and pheochromocytoma, retinoblastoma, carotid body tumor, cutaneous melanocarcinoma, intraocular melanocarcinoma, xeroderma pigmentosum, ataxia telangiectasia, Chediak-Higashi syndrome, albinism, Fanconi's aplastic anemia and Bloom's syndrome).

In another preferred embodiment, a Therapeutic of the present invention is administered to a human patient to prevent the progression to breast, colon, lung, pancreatic, or uterine cancer, or melanoma or sarcoma.

**Hyperproliferative and dysproliferative disorders**

In a preferred embodiment of the present invention, a Therapeutic is administered in the therapeutic or prophylactic treatment of hyperproliferative or benign dysproliferative disorders. The efficacy in treating or preventing hyperproliferative diseases or disorders of a Therapeutic of the present invention may be assayed by any method known within the art. Such assays include *in vitro* cell

proliferation assays, *in vitro* or *in vivo* assays using animal models of hyperproliferative diseases or disorders, or the like. Potentially effective Therapeutics may, for example, promote cell proliferation in culture or cause growth or cell proliferation in animal models in comparison to controls.

In accord, once a hyperproliferative disorder has been shown to be amenable to treatment by modulation of p27(Kip1)•FKBP-12 complex activity, the hyperproliferative disease or disorder may be treated or prevented by the administration of a Therapeutic that modulates p27(Kip1)•FKBP-12 complex formation (including supplying p27(Kip1)•FKBP-12 complex and/or the individual binding partners of a p27(Kip1)•FKBP-12 complex).

Specific embodiments of the present invention are directed to the treatment or prevention of cirrhosis of the liver (a condition in which scarring has overtaken normal liver regeneration processes); treatment of keloid (hypertrophic scar) formation causing disfiguring of the skin in which the scarring process interferes with normal renewal; psoriasis (a common skin condition characterized by excessive proliferation of the skin and delay in proper cell fate determination); benign tumors; fibrocystic conditions and tissue hypertrophy (*e.g.*, benign prostatic hypertrophy).

**Neurodegenerative disorders**

p27(Kip1) and its binding partner FKBP-12 have been implicated in the deregulation of cellular maturation and apoptosis, which are both characteristic of neurodegenerative disease. Accordingly, Therapeutics of the invention, particularly but not limited to those that modulate (or supply) p27(Kip1)•FKBP-12 complex activity, may be effective in treating or preventing neurodegenerative disease. Therapeutics of the present invention that modulate p27(Kip1)•FKBP-12 complex activity involved in neurodegenerative disorders can be assayed by any method known in the art for efficacy in treating or preventing such neurodegenerative diseases and disorders. Such assays include *in vitro* assays for regulated cell maturation or inhibition of apoptosis or *in vivo* assays using animal models of neurodegenerative diseases or disorders, or any of the assays described *infra*. Potentially effective Therapeutics, for example but not by way of limitation, promote regulated cell maturation and prevent cell apoptosis in culture, or reduce neurodegeneration in animal models in comparison to controls.

Once a neurodegenerative disease or disorder has been shown to be amenable to treatment by modulation of p27(Kip1)•FKBP-12 complex activity, that neurodegenerative disease or disorder can be treated or prevented by administration of a Therapeutic that modulates p27(Kip1)•FKBP-12 complex formation or activity, including supplying a p27(Kip1)•FKBP-12 complex or an uncomplexed binding partner, *e.g.*, p27(Kip1) and/or FKBP-12. Such diseases include all degenerative disorders involved with aging, especially osteoarthritis and neurodegenerative disorders.

**Disorders related to organ transplantation**

FKBP-12 has been implicated in disorders related to organ transplantation, in particular but not limited to organ rejection. Therapeutics of the invention, particularly those that modulate (or supply) p27(Kip1)•FKBP-12 complex activity, may be effective in treating or preventing diseases or disorders related to organ transplantation. Therapeutics of the invention (particularly Therapeutics that modulate the levels or activity of a p27(Kip1)•FKBP-12 complex) can be assayed by any method known in the art for efficacy in treating or preventing such diseases and disorders related to organ transplantation. Such assays include *in vitro* assays for using cell culture models as described *infra*, or *in vivo* assays using animal models of diseases and disorders related to organ transplantation, see *e.g.*, *infra*. Potentially effective Therapeutics, for example but not by way of limitation, reduce immune rejection responses in animal models in comparison to controls.

Accordingly, once diseases and disorders related to organ transplantation are shown to be amenable to treatment by modulation of p27(Kip1)•FKBP-12 complex activity or formation, such diseases or disorders can be treated or prevented by administration of a Therapeutic that modulates p27(Kip1)•FKBP-12 complex activity or formation (including supplying a p27(Kip1)•FKBP-12 complex or individual p27(Kip1) and/or FKBP-12 proteins).

**Cardiovascular Disease**

p27(Kip1) has been implicated in cardiovascular disorders, including in atherosclerotic plaque formation. Diseases such as cardiovascular disease, including cerebral thrombosis or hemorrhage, ischemic heart or renal disease, peripheral vascular disease, or thrombosis of other major vessel, and other diseases, including diabetes mellitus, hypertension, hypothyroidism, cholesterol ester storage disease, systemic lupus erythematosus, homocysteinemia, and familial protein or lipid processing diseases, and the like, are either directly or indirectly associated with atherosclerosis. Accordingly, Therapeutics of the invention, particularly those that modulate (or supply) p27(Kip1)•FKBP-12 complex activity or formation may be effective in treating or preventing atherosclerosis-associated diseases or disorders. Therapeutics of the invention (particularly Therapeutics that modulate the levels or activity of a p27(Kip1)•FKBP-12 complex) can be assayed by any method known in the art, including those described *infra*, for efficacy in treating or preventing such diseases and disorders.

A vast array of animal and cell culture models exist for processes involved in atherosclerosis. A limited and non-exclusive list of animal models includes knockout mice for premature atherosclerosis (Kurabayashi and Yazaki, 1996, Int. Angiol. 15: 187-194), transgenic mouse models of atherosclerosis (Kappel *et al.*, 1994, FASEB J. 8: 583-592), antisense oligonucleotide treatment of animal models (Callow, 1995, Curr. Opin. Cardiol. 10: 569-576), transgenic rabbit models for atherosclerosis (Taylor,

1997, Ann. N.Y. Acad. Sci 811: 146-152), hypercholesterolemic animal models (Rosenfeld, 1996, Diabetes Res. Clin. Pract. 30 Suppl.: 1-11), hyperlipidemic mice (Paigen *et al.*, 1994, Curr. Opin. Lipidol. 5: 258-264), and inhibition of lipoxygenase in animals (Sigal *et al.*, 1994, Ann. N.Y. Acad. Sci. 714: 211-224). In addition, *in vitro* cell models include but are not limited to monocytes exposed to low density lipoprotein (Frostegard *et al.*, 1996, Atherosclerosis 121: 93-103), cloned vascular smooth muscle cells (Suttles *et al.*, 1995, Exp. Cell Res. 218: 331-338), endothelial cell-derived chemoattractant exposed T cells (Katz *et al.*, 1994, J. Leukoc. Biol. 55: 567-573), cultured human aortic endothelial cells (Farber *et al.*, 1992, Am. J. Physiol. 262: H1088-1085), and foam cell cultures (Libby *et al.*, 1996, Curr Opin Lipidol 7: 330-335). Potentially effective Therapeutics, for example but not by way of limitation, reduce foam cell formation in cell culture models, or reduce atherosclerotic plaque formation in hypercholesterolemic mouse models of atherosclerosis in comparison to controls.

Accordingly, once an atherosclerosis-associated disease or disorder has been shown to be amenable to treatment by modulation of p27(Kip1)•FKBP-12 complex activity or formation, that disease or disorder can be treated or prevented by administration of a Therapeutic that modulates p27(Kip1)•FKBP-12 complex activity or formation including supplying a p27(Kip1)•FKBP-12 complex, or individual uncomplexed p27(Kip1) and/or FKBP-12 proteins.

**(6) Gene Therapy**

In a specific embodiment of the present invention, nucleic acids comprising a sequence that encodes p27(Kip1) and/or FKBP-12, or functional derivatives thereof, are administered to modulate p27(Kip1)•FKBP-12 complex function, by way of gene therapy. In more specific embodiments, a nucleic acid or nucleic acids encoding both p27(Kip1) and FKBP-12, or functional derivatives thereof, are administered by way of gene therapy. Gene therapy refers to therapy that is performed by the administration of a specific nucleic acid to a subject. In this embodiment of the present invention, the nucleic acid produces its encoded protein(s), which then serve to exert a therapeutic effect by modulating p27(Kip1)•FKBP-12 complex function. Any of the methodologies relating to gene therapy available within the art may be used in the practice of the present invention. See *e.g.*, Goldspiel, *et al.*, 1993. *Clin. Pharm.* 12: 488-505.

In a preferred embodiment, the Therapeutic comprises a p27(Kip1) and/or FKBP-12 nucleic acid that is part of an expression vector expressing both of the aforementioned proteins, or fragments or chimeric proteins thereof, within a suitable host. In a specific embodiment, such a nucleic acid possesses a promoter that is operably-linked to p27(Kip1) and FKBP-12 coding region(s), or, less preferably, two separate promoters linked to separate p27(Kip1) and FKBP-12 coding regions. Said promoter may be inducible or constitutive, and, optionally, tissue-specific. In another specific embodiment, a nucleic acid

molecule is used in which p27(Kip1) and FKBP-12 coding sequences (and any other desired sequences) are flanked by regions that promote homologous recombination at a desired site within the genome, thus providing for intra-chromosomal expression of p27(Kip1) and FKBP-12 nucleic acids. See *e.g.*, Koller & Smithies, 1989. *Proc. Natl. Acad. Sci. USA* 86: 8932-8935.

Delivery of the Therapeutic nucleic acid into a patient may be either direct (*i.e.*, the patient is directly exposed to the nucleic acid or nucleic acid-containing vector) or indirect (*i.e.*, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient). These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy. In a specific embodiment of the present invention, a nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This may be accomplished by any of numerous methods known in the art including, but not limited to, constructing said nucleic acid as part of an appropriate nucleic acid expression vector and administering the same in a manner such that it becomes intracellular (*e.g.*, by infection using a defective or attenuated retroviral or other viral vector; see U.S. Patent No. 4,980,286); directly injecting naked DNA; using microparticle bombardment (*e.g.*, a "Gene Gun®; Biolistic, DuPont); coating said nucleic acids with lipids; using associated cell-surface receptors/transfecting agents; encapsulating in liposomes, microparticles, or microcapsules; administering it in linkage to a peptide that is known to enter the nucleus; or by administering it in linkage to a ligand predisposed to receptor-mediated endocytosis (see *e.g.*, Wu & Wu, 1987. *J. Biol. Chem.* 262: 4429-4432), which can be used to "target" cell types that specifically express the receptors of interest, etc.

In another specific embodiment of the present invention, a nucleic acid-ligand complex may be produced in which the ligand comprises a fusogenic viral peptide designed so as to disrupt endosomes, thus allowing the nucleic acid to avoid subsequent lysosomal degradation. In yet another specific embodiment, the nucleic acid may be targeted *in vivo* for cell-specific endocytosis and expression, by targeting a specific receptor. See *e.g.*, PCT Publications WO 92/06180; WO93/14188 and WO 93/20221. Alternatively, the nucleic acid may be introduced intracellularly and incorporated within a host cell genome for expression by homologous recombination. See *e.g.*, Zijlstra, *et al.*, 1989. *Nature* 342: 435-438.

In yet another specific embodiment, a viral vector that contains p27(Kip1) and/or FKBP-12 nucleic acids is utilized. For example, retroviral vectors may be employed (see *e.g.*, Miller, *et al.*, 1993. *Meth. Enzymol.* 217: 581-599) that have been modified to delete those retroviral-specific sequences that are not required for packaging of the viral genome, with its subsequent integration into host cell DNA. p27(Kip1) and/or FKBP-12 (preferably both) nucleic acids may be cloned into a vector that facilitates delivery of the genes into a patient. See *e.g.*, Boesen, *et al.*, 1994. *Biotherapy* 6: 291-302; Kiem, *et al.*, 1994. *Blood* 83: 1467-1473. Additionally, adenovirus may be used as an especially efficacious "vehicle"

for the delivery of genes to the respiratory epithelia. Other targets for adenovirus-based delivery systems are liver, central nervous system, endothelial cells, and muscle. Adenoviruses also possess advantageous abilities to infect non-dividing cells. For a review see *e.g.*, Kozarsky & Wilson, 1993. *Curr. Opin. Gen. Develop.* 3: 499-503. Adenovirus-associated virus (AAV) has also been proposed for use in gene therapy. See *e.g.*, Walsh, *et al.*, 1993. *Proc. Soc. Exp. Biol. Med.* 204: 289-300.

An additional approach to gene therapy in the practice of the present invention involves transferring a gene into cells in *in vitro* tissue culture by such methods as electroporation, lipofection, calcium phosphate-mediated transfection, viral infection, or the like. Generally, the methodology of transfer includes the concomitant transfer of a selectable marker to the cells. The cells are then placed under selection pressure (*e.g.*, antibiotic resistance) so as to facilitate the isolation of those cells that have taken up, and are expressing, the transferred gene. Those cells are then delivered to a patient. In a specific embodiment, prior to the *in vivo* administration of the resulting recombinant cell, the nucleic acid is introduced into a cell by any method known within the art including, but not limited to: transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences of interest, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, and similar methodologies that ensure that the necessary developmental and physiological functions of the recipient cells are not disrupted by the transfer. See *e.g.*, Loeffler & Behr, 1993. *Meth. Enzymol.* 217: 599-618. The chosen technique should provide for the stable transfer of the nucleic acid to the cell, such that the nucleic acid is expressible by the cell. Preferably, said transferred nucleic acid is heritable and expressible by the cell progeny.

In preferred embodiments of the present invention, the resulting recombinant cells may be delivered to a patient by various methods known within the art including, but not limited to, injection of epithelial cells (*e.g.*, subcutaneously), application of recombinant skin cells as a skin graft onto the patient, and intravenous injection of recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells). The total amount of cells that are envisioned for use depend upon the desired effect, patient state, and the like, and may be determined by one skilled within the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and may be xenogeneic, heterogeneic, syngeneic, or autogeneic. Cell types include, but are not limited to, differentiated cells such as epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes and blood cells, or various stem or progenitor cells, in particular embryonic heart muscle cells, liver stem cells (International Patent Publication WO 94/08598), neural stem cells (Stemple and Anderson, 1992, *Cell 71*: 973-985), hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, and the like. In a preferred embodiment, the cells utilized for gene therapy are autologous to the patient.

In a specific embodiment in which recombinant cells are used in gene therapy, stem or progenitor cells that can be isolated and maintained *in vitro* may be utilized. Such stem cells include, but are not limited to, hematopoietic stem cells (HSC), stem cells of epithelial tissues, and neural stem cells (see *e.g.*, Stemple & Anderson, 1992. *Cell* 71: 973-985). With respect to HSCs, any technique that provides for the isolation, propagation, and maintenance *in vitro* of HSC may be used in this specific embodiment of the invention. As previously discussed, the HSCs utilized for gene therapy may, preferably, be autologous to the patient. When used, non-autologous HSCs are, preferably, utilized in conjunction with a method of suppressing transplantation immune reactions of the future host/patient. See *e.g.*, Kodo, *et al.*, 1984. *J. Clin. Invest.* 73: 1377-1384. In another preferred embodiment of the present invention, HSCs may be highly enriched (or produced in a substantially-pure form), by any techniques known within the art, prior to administration to the patient. See *e.g.*, Witlock & Witte, 1982. *Proc. Natl. Acad. Sci. USA* 79: 3608-3612.

**(7) Utilization of Anti-Sense Oligonucleotides**

In a specific embodiment of the present invention, p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex formation and function may be inhibited by the use of anti-sense nucleic acids for p27(Kip1) or FKBP-12, or most preferably, p27(Kip1) and FKBP-12. In addition, the present invention discloses the therapeutic or prophylactic use of nucleic acids (of at least six nucleotides in length) that are anti-sense to a genomic sequence (gene) or cDNA encoding p27(Kip1) and/or FKBP-12, or portions thereof. Such anti-sense nucleic acids have utility as Therapeutics that inhibit p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex formation or activity, and may be utilized in a therapeutic or prophylactic manner.

Another specific embodiment of the present invention discloses methodologies for inhibition of expression of p27(Kip1) and FKBP-12 nucleic acid sequences within a prokaryotic or eukaryotic cell, such as providing a cell with an therapeutically-effective amount of an anti-sense nucleic acid of p27(Kip1) and/or FKBP-12, or derivatives thereof.

The anti-sense nucleic acids of the present invention may be oligonucleotides that may either be directly administered to a cell or that may be produced *in vivo* by transcription of the exogenous, introduced sequences. In addition, the anti-sense nucleic acid may be complementary to either a coding (*i.e.*, exonic) and/or non-coding (*i.e.*, intronic) region of p27(Kip1) or FKBP-12 mRNAs. p27(Kip1) and FKBP-12 anti-sense nucleic acids are, at least, six nucleotides in length and are, preferably, oligonucleotides ranging from 6-200 nucleotides in length. In specific embodiments, the anti-sense oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The anti-sense oligonucleotides may be DNA or RNA (or chimeric mixtures, derivatives or

modified versions thereof), may be either single-stranded or double-stranded and may be modified at a base, sugar or phosphate backbone moiety.

In addition, said anti-sense oligonucleotide may include other associated functional groups, such as peptides, moieties that facilitate the transport of the oligonucleotide across the cell membrane, hybridization-triggered cross-linking agents, hybridization-triggered cleavage-agents, and the like. See *e.g.*, Letsinger, *et al.*, 1989. *Proc. Natl. Acad. Sci. U.S.A.* 86: 6553-6556; PCT Publication No. WO 88/09810. In a specific embodiment, p27(Kip1) and FKBP-12 antisense oligonucleotides comprise catalytic RNAs or ribozymes. See, *e.g.*, Sarver, *et al.*, 1990. *Science* 247: 1222-1225.

The anti-sense oligonucleotides of the present invention may be synthesized by standard methodologies known within the art including, but not limited to: (*i*) automated phosphorothioate-mediated oligonucleotide synthesis (see *e.g.*, Stein, *et al.*, 1988. *Nuc. Acids Res.* 16: 3209) or (*ii*) methylphosphonate oligonucleotides prepared by use of controlled pore glass polymer supports (see *e.g.*, Sarin, *et al.*, 1988. *Proc. Natl. Acad. Sci. U.S.A.* 85: 7448-7451).

In an alternative embodiment, p27(Kip1) and FKBP-12 antisense nucleic acids are produced intracellularly by transcription of an exogenous sequence. For example, a vector comprising a promoter functionally linked to the reverse complement of a desired gene, and the like, may be produced that (upon being taken up by the cell) is transcribed *in vivo,* thus producing an antisense nucleic acid (RNA) species. The aforementioned vector may either remain episomal or become chromosomally-integrated, so long as it can be transcribed to produce the desired antisense RNA. An origin of the vectors utilized may be derived from bacterial, viral, yeast or other sources known within the art that are utilized for replication and expression in mammalian cells. Expression of the sequences encoding p27(Kip1) and FKBP-12 antisense RNAs may be facilitated by any promoter known within the art to function in mammalian, preferably, human cells. Such promoters may be inducible or constitutive and include, but are not limited to: (*i*) the SV40 early promoter region; (*ii*) the promoter contained in the 3'-terminus long terminal repeat of Rous sarcoma virus (RSV); (*iii*) the Herpesvirus thymidine kinase promoter and (*iv*) the regulatory sequences of the metallothionein gene.

p27(Kip1) and FKBP-12 antisense nucleic acids may be utilized prophylactically or therapeutically in the treatment or prevention of disorders of a cell type that expresses (or preferably over-expresses) p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex. Cell types that express or over-express p27(Kip1) and FKBP-12 RNA may be identified by various methods known within the art including, but not limited to, hybridization with p27(Kip1) and FKBP-12-specific nucleic acids (*e.g.*, by Northern hybridization, dot blot hybridization, *in situ* hybridization) or by observing the ability of RNA from the specific cell type to be translated *in vitro* into p27(Kip1) and/or FKBP-12 by immunohistochemistry. In a preferred aspect, primary tissue from a patient may be assayed for

p27(Kip1) and/or FKBP-12 expression by, for example, immunocytochemistry or *in situ* hybridization, prior to actual treatment.

Pharmaceutical compositions of the present invention, comprising an effective amount of a p27(Kip1) and FKBP-12 antisense nucleic acid contained within a pharmaceutically-acceptable carrier, may be administered to a patient having a disease or disorder of a type that involves modified expression of p27(Kip1)•FKBP-12 complex, or of RNA or protein of the individual components of said complex. The amount of p27(Kip1) and/or FKBP-12 antisense nucleic acid that is effective in the treatment of a particular disorder or condition will be dependant upon the nature of the disorder or condition, and may be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity in *in vitro* systems and in useful animal model prior to testing and use in humans. In a specific embodiment, pharmaceutical compositions comprising p27(Kip1) and FKBP-12 antisense nucleic acids may be administered via liposomes, microparticles, or microcapsules or the like. See, *e.g.*, *supra*, and Leonetti, *et al.*, 1990. *Proc. Natl. Acad. Sci. U.S.A.* 87: 2448-2451.

**(8) p27(Kip1)•FKBP-12 Complex Assays**

The functional activity of p27(Kip1)•FKBP-12 complex (and derivatives, fragments, analogs and homologs thereof) may be assayed by a number of methods known in the art. For example, putative modulators (*e.g.*, inhibitors, agonists and antagonists) of p27(Kip1)•p27(Kip1) complex activity (*e.g.*, anti-p27(Kip1)•FKBP-12 complex antibodies, as well as p27(Kip1) or FKBP-12 antisense nucleic acids) may be assayed for their ability to modulate p27(Kip1)•FKBP-12 complex formation and/or activity.

**Immunoassays**

In a specific embodiment, immunoassay-based methodologies are provided wherein one is assaying for: (*i*) the ability to bind to, or compete with, wild-type p27(Kip1)•FKBP-12 complex or FKBP-12, or (*ii*) the ability to bind to an anti-p27(Kip1)•FKBP-12 complex antibody. These immunoassays include, but are not limited to, competitive and non-competitive assay systems utilizing techniques such as radioimmunoassays, enzyme linked immunosorbent assay (ELISA), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (*e.g.*, using colloidal gold, enzyme or radioisotope labels), complement fixation assays, Western blots, Northwestern blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), immunofluorescence assays, protein-A assays and immunoelectrophoresis assays, and the like. In one specific embodiment, antibody binding is detected directly by assaying for a label on a primary antibody. In another specific embodiment, binding of the

primary antibody is ascertained by detection of a secondary antibody (or reagent) that is specific for the primary antibody. In a further embodiment, the secondary antibody is labeled.

**Gene Expression Assays**

Expression of p27(Kip1) or FKBP-12 genes (from both endogenous genes and from incorporated recombinant DNA) may be detected using techniques known within the art including, but not limited to, Southern hybridization, Northern hybridization, restriction endonuclease mapping, DNA sequence analysis, and polymerase chain reaction amplification (PCR) followed by Southern hybridization or RNase protection (see *e.g.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY 1997, John Wiley and Sons, New York, NY) with probes specific for p27(Kip1) and FKBP-12 genes in various cell types.

In one specific embodiment of the present invention, Southern hybridization may be used to detect genetic linkage of p27(Kip1) and/or FKBP-12 gene mutations to physiological or pathological states. Numerous cell types, at various stages of development, may be characterized for their expression of p27(Kip1) and FKBP-12 (particularly the concomitant expression of p27(Kip1) and FKBP-12 within the same cells). The stringency of the hybridization conditions for Northern or Southern blot analysis may be manipulated to ensure detection of nucleic acids with the desired degree of relatedness to the specific probes used. See, *e.g.*, *supra*. Modification of these aforementioned methods, as well as other methods well-known within the art, may be utilized in the practice of the present invention.

**Binding Assays**

Derivatives, fragments, analogs and homologs of FKBP-12 may be assayed for binding to p27(Kip1) by any method known within the art including, but not limited to: (*i*) the modified yeast two hybrid assay system; (*ii*) immunoprecipitation with an antibody that binds to p27(Kip1) within a complex, followed by analysis by size fractionation of the immunoprecipitated proteins (*e.g.*, by denaturing or non-denaturing polyacrylamide gel electrophoresis); (*iii*) Western analysis; (*v*) non-denaturing gel electrophoresis, and the like. Alternatively, the aforementioned techniques may be modified to allow for the reverse analysis, whereby p27(Kip1) components bind to FKBP-12.

**Assays for Biological Activity**

A specific embodiment of the present invention provides a methodology for screening a derivative, fragment, analog or homolog of p27(Kip1) for biological activity, which is comprised of contacting said derivative, fragment, analog or homolog of p27(Kip1) with FKBP-12 and detecting complex formation between said derivative, fragment, analog or homolog of p27(Kip1) and FKBP-12; wherein the detection of the formation of said complex indicates that said p27(Kip1) derivative,

fragment, analog or homolog, possesses biological (*e.g.*, binding) activity. Similarly, an additional embodiment discloses a methodology for the screening a derivative, fragment, analog or homolog of FKBP-12 for biological activity comprising contacting said derivative, fragment, analog or homolog of said protein with p27(Kip1); and detecting complex formation between said derivative, fragment, analog or homolog of FKBP-12 and p27(Kip1); wherein detecting the formation of said complex indicates that said FKBP-12 derivative, fragment, analog, or homolog possesses biological activity.

**Modulation of p27(Kip1)•FKBP-12 Complex Activity**

The present invention provides methodologies relating to modulating the level or activity of a protein moiety that possesses the ability to participate in a p27(Kip1)•FKBP-12 complex, via the administration of a binding partner of that protein (or derivative, fragment, analog or homolog thereof). p27(Kip1) (and derivatives, fragments, analogs and homologs thereof) may be assayed for its ability to modulate the activity or levels of FKBP-12 by contacting a cell, or administering to an animal expressing the FKBP-12 gene, with p27(Kip1) protein, or, alternatively, with a nucleic acid encoding p27(Kip1) or an antibody that immunospecifically-binds p27(Kip1), or derivative, fragment, analog, or homolog thereof that contains the antibody binding domain, and measuring a change in FKBP-12 levels or activity, wherein said change in FKBP-12 levels or activity indicates that said p27(Kip1) possesses the ability to modulate FKBP-12 levels or activity. In another embodiment, FKBP-12 (and derivatives, fragments, analogs and homologs thereof) may be assayed for their ability to modulate the activity or levels of p27(Kip1) in an analogous manner.

**p27(KIP1)-Related Treatment Assays**

**Tumorigenesis**

p27(Kip1) plays a role in the control of cell proliferation and, therefore, of cell-transformation and tumorigenesis. The present invention discloses methodologies for screening p27(Kip1)•FKBP-12 complex (and derivatives, fragments, analogs and homologs, thereof) for the ability to alter cell proliferation, cell transformation and/or tumorigenesis *in vitro* and *in vivo*. For example, but not by way of limitation, cell proliferation may be assayed by measuring $^{3}$H-thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (*e.g.*, c-*fos*, c-*myc*) cell-cycle markers, and the like.

p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex (and derivatives, fragments, analogs and homologs, thereof) may also be screened for activity in inducing or inhibiting cell transformation (or the progression to malignant phenotype) *in vitro*. The proteins and protein complexes of the present

invention may be screened by contacting either cells with a normal phenotype (for assaying for cell transformation) or cells with a transformed phenotype (for assaying for inhibition of cell transformation) with the uncomplexed or complexed proteins of the present invention and examining said cells for acquisition or loss of characteristics associated with a transformed phenotype (*e.g.*, a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*) including, but not limited to, colony formation in soft agar, a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250 kDal cell-surface protein, and the like. See *e.g.*, Luria, *et al.*, 1978. GENERAL VIROLOGY, 3rd ed. (Wiley & Sons, New York, NY).

p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex (and derivatives, fragments, analogs and homologs, thereof) may also be screened for activity to promote or inhibit tumor formation *in vivo* in non-human test animal. A vast number of animal models of hyperproliferative disorders (*e.g.*, tumorigenesis and metastatic spread) are known within the art. See *e.g.*, Lovejoy, *et al.*, 1997. *J. Pathol.* 181: 130-135. In a specific embodiment of the present invention, the uncomplexed or complexed proteins of the present invention may be administered to a non-human test animal (preferably a test animal predisposed to develop a type of tumor), wherein the non-human test animal is subsequently examined for increased incidence of tumor formation in comparison with controls animals that were not administered the individual proteins or the protein complex of the present invention. Alternatively, the individual proteins and/or the protein complex may be administered to non-human test animals possessing tumors (*e.g.*, animals in which tumors have been induced by introduction of malignant, neoplastic, or transformed cells or by administration of a carcinogen) and subsequently examining the tumors within the test animals for tumor regression in comparison to controls. Accordingly, once a hyperproliferative disease or disorder has been shown to be amenable to treatment by modulation of p27(Kip1)•FKBP-12 complex activity, that disease or disorder may be treated or prevented by administration of a Therapeutic that modulates p27(Kip1)•FKBP-12 complex formation.

**Neurodegeneration**

Similarly, once a neurodegeneration disease or disorder has been shown to be amenable to treatment by modulation of p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity or formation, that disease or disorder can be treated or prevented by administration of a Therapeutic that modulates p27(Kip1)•FKBP-12 complex activity or formation, including supplying p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex. In a specific embodiment, p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex is administered to treat or prevent a neurodegenerative disease or disorder. p27(Kip1) has been implicated in the development and involution of all organs, including the

central nervous system. Casaccia-Bonnefil *et al.*, 1997, Genes and Dev. 11: 2335-2346. Accordingly, a p27(Kip1)•FKBP-12 complex or derivative, homolog, analog or fragment thereof, nucleic acid molecules encoding p27(Kip1) or FKBP-12, anti-p27(Kip1)•FKBP-12 complex antibodies, and other modulators of p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity or formation can be tested for activity in treating or preventing neurodegenerative disease in *in vitro* and *in vivo* assays.

In one embodiment, a Therapeutic of the invention can be assayed for activity in treating or preventing neurodegenerative disease by contacting a cultured cell that exhibits an indicator of a neurodegenerative disease *in vitro* with the Therapeutic and comparing the level of said indicator in the cell so contacted with the Therapeutic, with said level of said indicator in a cell not so contacted, wherein a lower level in said contacted cell indicates that the Therapeutic has activity in treating or preventing neurodegenerative disease. Specific examples of such cultured models for neurodegenerative disease include, but are not limited to, cultured rat endothelial cells from affected and nonaffected individuals (Maneiro *et al.*, 1997, Methods Find. Exp. Clin. Pharmacol. 19: 5-12); P19 murine embryonal carcinoma cells (Hung *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89: 9439-9443); and dissociated cell cultures of cholinergic neurons from nucleus basalis of Meynert (Nakajima *et al.*, 1985, Proc. Natl. Acad. Sci. USA 82: 6325-6329).

In another embodiment, a Therapeutic of the invention can be assayed for activity in treating or preventing neurodegenerative disease by administering the Therapeutic to a test animal that is predisposed to develop symptoms of a neurodegenerative disease, and measuring the change in said symptoms after administration of said Therapeutic, wherein a reduction in the severity of the symptoms of the neurodegenerative disease, or the prevention of the symptoms of the neurodegenerative disease, indicates that the Therapeutic has activity in treating or preventing said disease states. Such a test animal can be any one of a number of animal models known in the art for neurodegenerative disease. These models, including those for Alzheimer's Disease and mental retardation of trisomy 21, accurately mimic natural human neurodegenerative diseases. Farine, 1997, *Toxicol.* 119: 29-35. Examples of specific models include, but are not limited to, the partial trisomy 16 mouse (Holtzman *et al.*, 1996, Proc. *Natl. Acad. Sci. USA* 93: 13333-13338); bilateral nucleus basalis magnocellularis-lesioned rats (Popovic *et al.*, 1996, *Int. J. Neurosci.* 86: 281-299); the aged rat (Muir, 1997, *Pharmacol. Biochem. Behav.* 56: 687-696); the PDAPP transgenic mouse model of Alzheimer's disease (Johnson-Wood *et al.*, 1997, *Proc. Natl. Acad. Sci. USA 94*: 1550-1555); and experimental autoimmune dementia (Oron *et al.*, 1997, *J. Neural Transm.* Suppl. 49: 77-84).

**Autoimmune disease**

The p27(Kip1) binding partner FKBP-12 is implicated in autoimmune disease. Accordingly, p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex, or derivative, analog, or fragment thereof, nucleic acids encoding the p27(Kip1) and FKBP-12 proteins, or derivative, analogs or fragments thereof, or anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibodies, or other modulators of p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity or formation can be tested for activity in treating or preventing autoimmune disease in *in vitro* and *in vivo* assays.

In one embodiment of the present invention, a Therapeutic of the present invention can be assayed for activity in treating or preventing autoimmune disease by contacting a cultured cell that exhibits an indicator of an autoimmune reaction *in vitro* with the Therapeutic, and comparing the level of said indicator in the cell so contacted with the Therapeutic with said level of the indicator in a cell not so contacted, wherein a lower level in said contacted cell indicates that the Therapeutic has activity in treating or preventing autoimmune disease. Cell models that can be used for such assays include, but are not limited to, leukocyte and other synovial cells that secrete chemokines mediating inflammation (Kunkel *et al.*, 1996, *J. Leukoc. Biol.* 59: 6-12); cerebrospinal fluid cells from animal models of multiple sclerosis (Norga *et al.*, 1995, *Inflamm. Res.* 44: 529-534); macrophages in experimental autoimmunoneuritis, a model of Guillain-Barre Disease (Bai *et al.*, 1997, J. *Neuroimmunol.* 76: 177-184); CD40/CD40L assays in monocytes (Laman *et al.*, 1996, *Crit. Rev. Immunol.* 16: 59-108); lymphocyte cultures for *lpr* mice (Nagata, 1996, *Prog. Mol. Subcell. Biol.* 16: 87-103); and cultured thyrocytes in spontaneous murine autoimmune thyroiditis (Green *et al.*, 1996, *Endocrinol* 137: 2823-32).

In another embodiment, a Therapeutic of the present invention can be assayed for activity in treating or preventing autoimmune disease by administering said Therapeutic to a test animal that exhibits an autoimmune reaction or, alternatively, does not exhibit an autoimmune reaction but is subsequently challenged with an agent that elicits an autoimmune reaction, and measuring the change in the autoimmune reaction after the administration of said Therapeutic, wherein a reduction in said autoimmune reaction or a prevention of said autoimmune reaction indicates that the Therapeutic has activity in treating or preventing an autoimmune disease.

A number of animal models of autoimmune disease are known in the art. These models, including those for arthritis, systemic lupus erythematosus, diabetes, thyroiditis, encephalitis etc., accurately mimic natural human autoimmune diseases. Farine, 1997, *Toxicol.* 119: 29-35. Examples of specific models include, but are not limited to, experimental allergic encephalomyelitis for multiple sclerosis (Brabb *et al.*, 1997, *J. Immunol.* 159: 497-507); thyroglobulin-induced experimental thyroiditis (Bhatia *et al.*, 1996 *Proc Soc Exp Biol Med.* 213: 294-300); multiple organ-localized autoimmune disease, *e.g.*, thyroiditis and gastritis in BALB/c nu/nu mice receiving rat thymus grafts under their renal

capsules (Taguchi and Takahashi, 1996, *Immunol* 89: 13-19); virus-induced autoimmune diseases such as insulin-dependent diabetes mellitus (Oldstone and von Herrath, 1996 *APMIS*. 104: 689-97. Review), experimental autoimmune encephalomyelitis (Encinas *et al.*, 1996, *J. Neurosci. Res*. 45: 655-669); experimental autoimmune labyrinthitis; Freund's-adjuvant induced rheumatoid arthritis and inbred mouse strains that develop systemic lupus erythematosus, rheumatoid arthritis, graft-vs-host disease, and diabetes (Humphryes-Beher, 1996, *Adv. Dent. Res*. 10: 73-75); and autoimmune hepatitis (Meyer zum Buschenfelde and Dienes, 1996, *Virchows Arch*. 429: 1-12).

Similarly, once an organ transplantation disease or disorder has been shown to be amenable to treatment by modulation of p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity or formation, that disease or disorder can be treated or prevented by administration of a Therapeutic that modulates said p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity or formation. In a specific embodiment, p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex is administered to treat or prevent organ transplantation related diseases or disorders.

**Atherosclerosis**

p27(Kip1) has been implicated in atherosclerosis as well. The major macrophage colony stimulating factor (MCSF), which is present in atherosclerotic plaques, is required for successful downregulation of p27(Kip1) before cell cycling. Antonov *et al.*, 1997, *J. Clin. Invest*. 99: 2867-2876. A p27(Kip1) protein, a FKBP-12 protein, and/or a p27(Kip1)•FKBP-12 complex, or a derivative, analog or fragment thereof, a nucleic acids encoding a p27(Kip1) or FKBP-12 protein or a derivative, analog or fragment, or an anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibodies, or other modulators of said p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity or formation, can be tested for activity in treating or preventing atherosclerosis in *in vitro* and *in vivo* assays. Accordingly, Therapeutics herein, particularly those that modulate (or supply) p27(Kip1), FKBP-12, and/or p27(Kip1)•FKBP-12 complex activity or formation, may be effective in treating or preventing atherosclerosis-associated diseases or disorders. Therapeutics of the invention can be assayed by any method known in the art for efficacy in treating or preventing such diseases and disorders.

In one embodiment, a Therapeutic of the present invention can be assayed for activity in treating or preventing atherosclerosis and associated diseases by contacting a cultured cell that exhibits an indicator of an atherosclerosis-associated disease *in vitro* with the Therapeutic, and comparing the level of said indicator in the cell so contacted with the Therapeutic, with said level of the indicator in a cell not so contacted, wherein a lower level in said contacted cell indicates that the Therapeutic has activity in treating or preventing atherosclerosis-associated disease. Specific examples of such cultured models for atherosclerosis and associated diseases include, but are not limited to, monocytes exposed to low density

lipoprotein (Frostegard *et al.*, 1996, *Atherosclerosis* 121: 93-103), cloned vascular smooth muscle cells (Suttles *et al.*, 1995, *Exp. Cell Res.* 218: 331-338), endothelial cell-derived chemoattractant exposed T cells (Katz *et al.*, 1994, *J. Leukoc. Biol.* 55: 567-573), cultured human aortic endothelial cells (Farber *et al.*, 1992, *Am. J. Physiol.* 262: H1088-1085), and foam cell cultures (Libby *et al.*, 1996, *Curr. Opin. Lipidol.* 7: 330-335).

In another embodiment, a Therapeutic of the present invention can be assayed for activity in treating or preventing atherosclerosis-associated diseases by administering the Therapeutic to a test animal that exhibits symptoms of an atherosclerosis-associated disease or that is predisposed to develop symptoms of said disease; and measuring the change in said symptoms of the atherosclerosis-associated disease after administration of said Therapeutic, wherein a reduction in the severity of the symptoms of the said disease, or prevention of the symptoms of the same, indicates that the Therapeutic has activity in treating or preventing atherosclerosis-associated disease. Such a test animal can be any one of a number of animal models known in the art for atherosclerosis-associated disease. A limited and non-exclusive list of animal models includes knockout mice for premature atherosclerosis (Kurabayashi and Yazaki, 1996, *Int. Angiol.* 15: 187-194), transgenic mouse models of atherosclerosis (Kappel *et al.*, 1994, *FASEB J.* 8: 583-592), antisense oligonucleotide treatment of animal models (Callow, 1995, *Curr. Opin. Cardiol.* 10: 569-576), transgenic rabbit models for atherosclerosis (Taylor, 1997, *Ann. N.Y. Acad. Sci* 811:146-152), hypercholesterolemic animal models (Rosenfeld, 1996, *Diabetes Res. Clin. Pract.* 30 Suppl.:1-11), hyperlipidemic mice (Paigen *et al.*, 1994, *Curr. Opin. Lipidol.* 5: 258-264), and inhibition of lipoxygenase in animals (Sigal *et al.*, 1994, *Ann. N.Y. Acad. Sci.* 714: 211-224).

**Membranous nephropathy**

p27(Kip1) has been implicated in membranous nephropathy. A model of membranous nephropathy, which shows aberrant expression of visceral glomerular epithelial cells, demonstrates a marked upregulation of p27(Kip1). See, *e.g.*, Shankland *et al.*, 1997, *Kidney Intl* 52: 404-413. A p27(Kip1) or FKBP-12 protein, and/or a p27(Kip1)•FKBP-12 complex, or a derivative, analog or fragment thereof, or nucleic acids encoding a p27(Kip1) or FKBP-12 protein or derivative, analog or fragment, or anti-p27(Kip1), anti-FKBP-12, and/or anti-p27(Kip1)•FKBP-12 complex antibodies, or other modulators of p27(Kip1)•FKBP-12 complex activity or formation can be tested for activity in treating or preventing nephropathy in *in vitro* and *in vivo* assays, as described, *supra*.

**Protein-Protein Interaction Assays**

The present invention discloses methodologies for assaying and screening derivatives, fragments, analogs and homologs of FKBP-12 for binding to p27(Kip1). The derivatives, fragments,

analogs and homologs of the FKBP-12 that interact with p27(Kip1) may be identified by means of a yeast two hybrid assay system (see *e.g.*, Fields & Song, 1989. *Nature* 340: 245-246) or; preferably, a modification and improvement thereof, as described in U.S. Patent Applications Serial Nos. 08/663,824 (filed June 14, 1996) and 08/874,825 (filed June 13, 1997), to Nandabalan, *et al.*, and that are incorporated by reference herein in their entireties.

The identification of interacting proteins by the improved yeast two hybrid system is based upon the detection of the expression of a reporter gene (hereinafter "Reporter Gene"), the transcription of which is dependent upon the reconstitution of a transcriptional regulator by the interaction of two proteins, each fused to one half of the transcriptional regulator. The bait p27(Kip1) (or derivative, fragment, analog or homolog) and prey protein (proteins to be tested for ability to interact with the bait protein) are expressed as fusion proteins to a DNA-binding domain, and to a transcriptional regulatory domain, respectively, or *vice versa*. In a specific embodiment of the present invention, the prey population may be one or more nucleic acids encoding mutants of FKBP-12 (*e.g.*, as generated by site-directed mutagenesis or another method of producing mutations in a nucleotide sequence). The prey populations are proteins encoded by DNA (*e.g.*, cDNA, genomic DNA or synthetically generated DNA), said DNAs derived either from a specific gene of choice, or from cDNA libraries obtain from a cell type of interest. For example, the populations may be expressed from chimeric genes comprising cDNA sequences derived from a non-characterized sample of a population of cDNA from mammalian RNA. In another specific embodiment, recombinant biological libraries expressing random peptides may be used as the source of prey nucleic acids.

The present invention discloses methods for the screening for inhibitors of FKBP-12. In brief, the protein-protein interaction assay may be performed as previously described herein, with the exception that it is performed in the presence of one or more candidate molecules. A resulting increase or decrease in Reporter Gene activity, in relation to that which was present when the one or more candidate molecules are absent, indicates that the candidate molecule exerts an effect on the interacting pair. In a preferred embodiment, inhibition of the protein interaction is necessary for the yeast cells to survive, for example, where a non-attenuated protein interaction causes the activation of the *URA3* gene, causing yeast to die in medium containing the chemical 5-fluoroorotic acid. See *e.g.*, Rothstein, 1983. *Meth. Enzymol.* 101: 167-180.

In general, the proteins comprising the bait and prey populations are provided as fusion (chimeric) proteins, preferably by recombinant expression of a chimeric coding sequence containing each protein contiguous to a pre-selected sequence. For one population, the pre-selected sequence is a DNA-binding domain that may be any DNA-binding domain, so long as it specifically recognizes a DNA sequence within a promoter (*e.g.*, a transcriptional activator or inhibitor). For the other population, the

pre-selected sequence is an activator or inhibitor domain of a transcriptional activator or inhibitor, respectively. The regulatory domain alone (not as a fusion to a protein sequence) and the DNA-binding domain alone (not as a fusion to a protein sequence) preferably, do not detectably interact, so as to avoid false-positives in the assay. The assay system further includes a reporter gene operably linked to a promoter that contains a binding site for the DNA-binding domain of the transcriptional activator (or inhibitor). Accordingly, in the practice of the present invention, the binding of p27(Kip1) fusion protein to a prey fusion protein leads to reconstitution of a transcriptional activator (or inhibitor), which concomitantly activates (or inhibits) expression of the Reporter Gene.

In a specific embodiment, the present invention discloses a methodology for detecting one or more protein-protein interactions comprising the following steps: (*i*) recombinantly-expressing p27(Kip1) (or a derivative, fragment, analog or homolog thereof) in a first population of yeast cells of a first mating type and possessing a first fusion protein containing p27(Kip1) sequence and a DNA-binding domain; wherein said first population of yeast cells contains a first nucleotide sequence operably-linked to a promoter that is "driven" by one or more DNA-binding sites recognized by said DNA-binding domain such that an interaction of said first fusion protein with a second fusion protein (comprising a transcriptional activation domain) results in increased transcription of said first nucleotide sequence; (*ii*) negatively selecting to eliminate those yeast cells in said first population in which said increased transcription of said first nucleotide sequence occurs in the absence of said second fusion protein; (*iii*) recombinantly expressing in a second population of yeast cells of a second mating type different from said first mating type, a plurality of said second fusion proteins; wherein said second fusion protein is comprised of a sequence of a derivative, fragment, analog or homolog of a FKBP-12 and an activation domain of a transcriptional activator, in which the activation domain is the same in each said second fusion protein; (*iv*) mating said first population of yeast cells with said second population of yeast cells to form a third population of diploid yeast cells, wherein said third population of diploid yeast cells contains a second nucleotide sequence operably linked to a promoter "driven" by a DNA-binding site recognized by said DNA-binding domain such that an interaction of a first fusion protein with a second fusion protein results in increased transcription of said second nucleotide sequence, in which the first and second nucleotide sequences can be the same or different and (*v*) detecting said increased transcription of said first and/or second nucleotide sequence, thereby detecting an interaction between a first fusion protein and a second fusion protein.

In a preferred embodiment, the bait (a p27(Kip1) sequence) and the prey (a library of chimeric genes) are combined by mating the two yeast strains on solid media for a period of approximately 6-8 hours. In a less preferred embodiment, the mating is performed in liquid media. The resulting diploids contain both types of chimeric genes (*i.e.*, the DNA-binding domain fusion and the activation domain fusion). After an interactive population is obtained, the DNA sequences encoding the pairs of interactive

proteins are isolated by a method wherein either the DNA-binding domain hybrids or the activation domain hybrids are amplified, in separate reactions. Preferably, the amplification is carried out by polymerase chain reaction (PCR; see *e.g.*, Innis, *et al.*, 1990. PCR PROTOCOLS, Academic Press, Inc., San Diego, CA) utilizing pairs of oligonucleotide primers specific for either the DNA-binding domain hybrids or the activation domain hybrids. The PCR amplification reaction may also be performed on pooled cells expressing interacting protein pairs, preferably pooled arrays of interactants. Other amplification methods known within the art may also be used including, but not limited to, ligase chain reaction; Qβ-replicase or the like. See *e.g.*, Kricka, *et al.*, 1995. MOLECULAR PROBING, BLOTTING, AND SEQUENCING, Academic Press, New York, NY.

In an additional embodiment of the present invention, the plasmids encoding the DNA-binding domain hybrid and the activation domain hybrid proteins may also be isolated and cloned by any of the methods well-known within the art. For example, but not by way of limitation, if a shuttle (yeast to *E. coli*) vector is used to express the fusion proteins, the genes may be subsequently recovered by transforming the yeast DNA into *E. coli* and recovering the plasmids from the bacteria. See *e.g.*, Hoffman, *et al.*, 1987. *Gene* 57: 267-272.

**Pharmaceutical Compositions**

The invention provides methods of treatment and prophylaxis by the administration to a subject of an pharmaceutically-effective amount of a Therapeutic of the invention. In a preferred embodiment, the Therapeutic is substantially purified and the subject is a mammal, and most preferably, human.

Formulations and methods of administration that can be employed when the Therapeutic comprises a nucleic acid are described *supra*. Various delivery systems are known and can be used to administer a Therapeutic of the present invention including, but not limited to: (*i*) encapsulation in liposomes, microparticles, microcapsules; (*ii*) recombinant cells capable of expressing the Therapeutic; (*iii*) receptor-mediated endocytosis (see, *e.g.*, Wu & Wu, 1987. *J. Biol. Chem.* 262: 4429-4432); (*iv*) construction of a Therapeutic nucleic acid as part of a retroviral or other vector, and the like.

Methods of administration include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The Therapeutics of the present invention may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically-active agents. Administration can be systemic or local. In addition, it may be advantageous to administer the Therapeutic into the central nervous system by any suitable route, including intraventricular and intrathecal injection. Intraventricular injection may be facilitated by an intraventricular catheter attached

to a reservoir (*e.g.*, an Ommaya reservoir). Pulmonary administration may also be employed by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. It may also be desirable to administer the Therapeutic locally to the area in need of treatment. This may be achieved by, for example, and not by way of limitation, by local infusion during surgery, by topical application, by injection, by means of a catheter, by means of a suppository, or by means of an implant. In a specific embodiment, administration may be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment of the present invention, the Therapeutic may be delivered in a vesicle, in particular a liposome. See *e.g.*, Langer, 1990. *Science* 249: 1527-1533. In yet another embodiment, the Therapeutic can be delivered in a controlled release system including, but not limited to, a delivery pump (see *e.g.*, Saudek, *et al.*, 1989. *New Engl. J. Med.* 321: 574) and a semi-permeable polymeric material (see *e.g.*, Howard, *et al.*, 1989. *J. Neurosurg.* 71: 105). Additionally, the controlled release system can be placed in proximity of the therapeutic target (*e.g.*, the brain), thus requiring only a fraction of the systemic dose. See, *e.g.*, Goodson, In: MEDICAL APPLICATIONS OF CONTROLLED RELEASE, CRC Press, Bocca Raton, FL (1984).

In a specific embodiment, where the Therapeutic is a nucleic acid encoding a protein, the Therapeutic nucleic acid may be administered *in vivo* to promote expression of its encoded protein by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular (*e.g.*, via a retroviral vector, direct injection, use of microparticle bombardment, coating with lipids or cell-surface receptors or transfecting agents, or administering it in linkage to a homeobox-like peptide that is known to enter the nucleus (see *e.g.*, Joliot, *et al.*, 1991. *Proc. Natl. Acad. Sci. USA* 88: 1864-1868), and the like. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated into host cell DNA for expression, *e.g.*, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically-effective amount of Therapeutic, and a pharmaceutically acceptable carrier. As utilized herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government, or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals and, more particularly, in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered and includes, but is not limited, to such sterile liquids as water and oils.

The amount of the Therapeutic of the invention that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and may be determined by standard clinical techniques by those of average skill within the art. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be

employed in the formulation will also depend on the route of administration and the overall seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and of each patient's circumstances. However, suitable dosage ranges for intravenous administration of a Therapeutics herein are generally about 20-500 micrograms (μg) of active compound per kilogram (kg) body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 picograms (pg)/kg body weight to 1 milligram (mg)/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The present invention also provides a pharmaceutical pack or kit, comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions and Therapeutics of the present invention. Optionally associated with such container(s) may be a notice in the form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceutical or biological products, which notice reflects approval by the agency of manufacture, use, or sale for human administration.

## *SPECIFIC EXAMPLES*

### Identification and Specificity of p27(Kip1)•FKBP-12

A modified, improved yeast two hybrid system was used to identify protein interactions for the cell cycle protein cyclin dependent kinase (CDK2). Yeast is a eukaryote, and therefore any intermolecular protein interactions detected in this system are likely to occur under physiological conditions in mammalian cells. Chien *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* 88: 9578-9581. One of the identified isolates (prey) was the known p27(Kip1) nucleic acid sequence (GenBank Accession Number U10906), starting from base 127-597 (Figure 1, SEQ ID NO:1 and SEQ ID NO:2). The interaction between CDK2 and p27(Kip1) has been described before. Kwon *et al.*, 1996 *Biochem. Biophys. Res. Comm.* 220: 703-709. The nucleic acid sequence and corresponding amino acid sequence of p27(Kip1) are shown in Figure 1.

### Identification and specificity of p27(Kip1)•FKBP-12 interaction

In a matrix-mating assay, p27(Kip1), CDK2, FKBP-12, and other proteins were inserted into complementary (a and alpha) mating types of yeast using methods known in the art. Mating was carried out to express both vector constructs within the same yeast cells, thus allowing interaction to occur. Interaction between the domains led to transcriptional activation of reporter genes containing *cis*-binding

elements for Gal4. The reporter genes encoding the indicator protein β-galactosidase, and metabolic markers for uracil and histidine auxotrophy, were included in specific fashion in one or the other of the yeast strains used in the mating. In this way, yeast were selected for successful mating, expression of both fusion constructs, and expression of p27(Kip1)-interacting proteins and the interaction of both fusion proteins.

The p27(Kip1) cDNA was obtained from a commercial fetal brain cDNA library of 3.5 x $10^6$ independent isolates (Clontech #HL4029AH, Palo Alto, CA). The library was synthesized from Xho 1-dT15 primed fetal brain mRNA (from five male/female 19-22 week fetuses) that was directionally cloned into pACT2, a yeast Gal4 activation domain cloning vector including the *LEU2* gene for selection in yeast deficient in leucine biosynthesis.

FKBP-12 was amplified from the Clontech pACT2 library by PCR using the forward primer 5'-GGACTAGGCCGAGGTGGCC**ATGGGAGTGCA**GGTGG**AAACCATC**-3' [SEQ ID NO:5] and the reverse primer 5'-GGACTAGGCCTCCTGGGCC**TCATTCCAGTTTTAGAAGCTCCAC**-3' [SEQ ID NO:6] by standard techniques (the nucleotides illustrated in **bold** refer to FKBP-12 sequences, GenBank Accession No. X55741). The fragment was cloned into the SfiI site of the vector pAS, constructed by introducing an SfiI-containing polylinker into the vector pAS2-1 (Clontech, Palo Alto, CA). This vector is a yeast DNA-binding domain cloning vector that contains the *TRP1* gene for selection in yeast strains deficient in tryptophan biosynthesis. The *FKBP-12* sequence was confirmed by nucleic acid sequencing to confirm that PCR amplification reproduced an accurate copy of the *FKBP-12* sequence. This test determined that as predicted, the sequence encoded an interacting domain identical to human *FKBP-12*.

**Test for the specificity of p27(Kip1)•FKBP-12 interaction**

p27(Kip1) was transformed by lithium acetate/polyethylene glycol transformation (Ito *et al.*, 1983, *J. Bacteriol.* 153: 163-168) into the yeast strain N106' (mating type a, *ura3*, *his3*, *ade2*, *trp1*, *leu2*, *gal4*, *gal80*, *cyh'*, *Lys2*::$GAL1_{UAS}$-$HIS3_{TATA}$-*HIS3*, *ura 3*::$GAL1_{UAS}$-$GAL_{TATA}$-*lacZ*), while the coding sequences of FKBP-12 were transformed into the yeast strain YULH (mating type alpha, *ura3*, *his3*, *lys2*, *trp1*, *leu2*, *gal4*, *gal80*, *GAL1-URA3*). The two transformed populations were then mated using standard methods in the art. Sherman *et al.*, eds., 1991, GETTING STARTED WITH YEAST, Vol. 194, Academic Press, New York. Briefly, cells were grown until mid-to-late log phase on media that selected for the presence of the appropriate plasmids. The two mating strains, alpha and a, were then, diluted in YAPD media, filtered onto nitrocellulose membranes, and incubated at 30° C for 6-8 hours. The cells were then transferred to media selective for the desired diploids, *i.e.*, yeast harboring reporter genes for β-galactosidase, uracil auxotrophy, and histidine auxotrophy, and expression of the vectors encoding the bait and prey. The mating products were plated on SC (synthetic complete) (Sambrook *et al.*, 1989, A

LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Press, New York) media lacking adenine and lysine (to select for successful mating), leucine and tryptophan (to select for expression of genes encoded by both plasmids), and uracil and histidine (to select for protein interactions). This medium is herein referred to as SCS medium, for SC Selective medium.

Selected clones were tested for expression of β-galactosidase to confirm the formation of a p27(Kip1)•FKBP-12 complex. Filter-lift β-galactosidase assays were performed as modified from the protocol of Breeden and Nasmyth, 1985, *Cold Spring Harbor Quant. Biol.* 50: 643-650. Colonies were patched onto SCS plates, grown overnight, and replica plated onto nitrocellulose filters. The filters were then assayed for β-galactosidase activity as per Breeden and Nasmyth, 1985, *Cold Spring Harbor Quant. Biol.* 50: 643-650. Colonies that were positive turned a visible blue.

To test for the specificity of p27(Kip1)•FKBP-12 interaction, two general tests were first performed. In the first instance, YULH cells expressing FKBP-12 were created and plated on SC (synthetic complete) -Ura plates, grown for 1-2 days, and examined for growth. No growth was found for FKBP-12, confirming that it is not a "self-activating" protein, that is, FKBP-12 requires interaction with a second protein domain for a functional activation complex. In the second instance, plasmids containing p27(Kip1) inserts were transformed into strain N106' (mating type alpha) and mated with yeast strain YULH (mating type a) expressing either CDK2, FKBP-12, or certain other proteins. Promiscuous binders, that is, insert products able to bind with many other proteins in a non-specific fashion, would interact non-specifically with non-CDK2 domains, and would be discarded as non-specific interactants. p27(Kip1) complexed specifically with FKBP-12, but not with trk oncogene (GenBank Accession No. X03541), nor with cyclophilin B (GenBank Accession No. M60857) or the vector-control. As illustrated in Figure 3, the intersection of the p27(Kip1) column with the FKBP-12 row indicates growth, *i.e.*, a positive interaction. In contrast, the intersection of the p27(Kip1) column with the rows for trk oncogene (trk), cyclophilin B (CYC-B) and vector-control indicates no growth, *i.e.*, no protein interaction. The known interaction between p27(Kip1) and CDK2 was confirmed, as shown in Figure 3 (intersection of column 1, row 1).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entirety.

## EQUIVALENTS

From the foregoing detailed description of the specific embodiments of the invention, it should be apparent that unique compositions and methods of use for p27(Kip), FKBP-12, and p27(Kip)•FKBP-12 complexes have been described. Although particular embodiments have been disclosed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims which follow. In particular, it is contemplated by the inventor that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims. For instance, the choice of disease states in which p27(Kip), FKBP-12, and p27(Kip)•FKBP-12 complexes provide utility through diagnosis, screening, treatment of various diseases and disorders is believed to be a matter of routine for a person of ordinary skill in the art with knowledge of the embodiments described herein.